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Interleukin-37 isoform A (IL-37a) is a novel regulator of immune cell function

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Abstract

Background: Interleukin (IL)-37 is a newly identified member of IL-1 cytokine family, and has five isoforms (IL-37a-e). While the function of IL-37b has been well studied as an important immunosuppressive cytokine that plays an anti-inflammatory role in several diseases, the functions of other IL-37 isoforms are largely unknown. The five IL-37 isoforms are varying in protein sequences, induction and tissue distribution, suggesting that the isoforms may differ in function. This study was focused on the IL-37a isoform, with IL-37b as a control. This was because our preliminary results showed that IL-37a expression is highly inducible in human macrophages by inflammatory stimuli compared to other IL-37 isoforms, suggesting that IL-37a may play an important role in inflammatory response. Furthermore, IL-37a protein is different from other IL-37 isoforms, in particular the N- terminal which contains a nuclear localisation sequence (NLS) and an elastase cleave site, suggesting that IL-37a may have unique biochemical features and functions.

Hypothesis: Based on the differences at the N-terminal, IL-37a and IL-37b isoforms may differ in cellular location and function. Based on protein sequence similarities with IL-37b at the C-terminal, which contains the IL-1 domain for receptor binding and signalling. IL-37a may be also bioactive by signalling via the same receptor as IL-37b.

Aim: To address the hypothesis, I set out the following aims: 1) to produce recombinant IL-37a and IL-37b proteins and compare their differences in protein levels (Chapter 3). 2) To investigate whether IL-37a is bioactive and what is the difference between IL-37a and b in the regulation of Toll-like receptor (TLR)-induced inflammatory response in immune cells (Chapter 4). 3) To explore the molecular mechanism by which IL-37a and b differently regulate inflammatory gene profiles and signalling pathways by transcriptomics.

Methods: To investigate their effect and difference in bioactivity, I produced recombinant IL-37a and b in *Escherichia coli* (*E.coli*) system and purified by affinity chromatography and gel filtration (Chapter 3). The effect of IL-37a and IL-37b on the regulation of TLR-induced inflammatory cell function been determined and

compared in macrophages and in B cells in vitro (Chapter 4). The B cells from IL-37a transgenic (IL-37a-tg) mice and wild type (wt) control mice were used for studying the effect of IL-37a on B cell functions (Chapter 4). The microarray and bioinformatics analysis was used to unravel the differences between IL-37a and b in term of genes regulation and signalling pathways in lipopolysaccharide (LPS)-stimulated spleenocytes from IL37a and b transgenic and wt control mice (Chapter 5).

Results:

i) Recombinant IL-37a and b proteins were successfully expressed in *E.coli* and purified by Nickel affinity chromatography, gel filtration and LPS removal. The proteins were used for crystallisation and biological study. The difference between IL-37a and b in protein and biochemical features was compared (Chapter 3).

ii) IL-37 isoforms expressions are differently regulated in immune cells, depending on the activation conditions, dose, and time of the stimuli. The IL-37a is one of the highly induced isoforms. By using several biological approaches, including IL-37a recombinant protein, ribonucleic acid (RNA) knockdown, and transgenic cells, my results demonstrated that IL-37a is bioactive. It is generally immunosuppressive and can regulate the expression of several TLR-induced inflammatory cytokines in macrophage and monocytes. IL-37a and IL-37b share functional similarity in the tested experimental conditions, suppress the same cytokine expressions, and full length IL-37a (FL-IL-37a) is more effective than the full length IL-37b (FL-IL-37b) in the suppression of LPS-induced IL-6 production in macrophages. Unexpectedly, both isoforms can increase B-cell antibody production and proliferation. The effect of IL-37a is more pronounced in mice than human B cells. This may be due to the difference between mice and human in the regulation of IL-37 and receptor expressions. Thus, IL-37a is a novel immunoregulatory cytokine of IL-37 family (Chapter 4).

iii) My transcriptomic analysis results revealed that IL-37a has a broad regulatory effect on global gene expression, by enhancing the expression of 2881 genes and down regulating the expression of 2905 genes. IL-37a differs from IL-37b in gene regulation; IL-37a specifically inhibited 1182 and enhanced 1525 genes; in

addition, common regulated genes between the two IL-37 isoforms were also identified, explaining the functional similarity between the two isoforms.

IL-37a and IL-37b control LPS/TLR4 signalling transduction through inhibiting of the co-receptor cluster of differentiation-14 (CD-14) and myeloid differentiation primary response gene-88 (MYD88) and toll/interleukin-1 receptor adaptor protein (TIRAP). The IL-37a also induces the expression of LPS-binding proteinphospholipid transfer protein (PLTP) to block LPS function. Both isoforms suppress the downstream TLR signalling, including Mitogen-activated protein kinase (MAPKs), phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin (mTOR), nuclear factor kappa-light-chain (NF- κ B) and Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways. However, both isoforms can also act independently in gene modulation and control TLR signalling, IL-37a supresses the expression of interferon response factors (IRF7) and interferon-beta-1 (IFN-B1) in the TIR-domain-containing adapter-inducing interferon-B (TRIF)-dependent pathway, on the other hand, IL-37b inhibited IRF3, together, this may lead to the down regulation of type I IFN production.

My results showed that IL-37a increased the expression of several anti-inflammatory immunoregulators, including peroxisome proliferator-activated receptors (PPAR γ) which inhibits TLR4-mediated inflammations and type II cytokine IL-13; and both IL-37 isoforms enhanced IL-4 receptor as well. Altogether, these effects may impair the LPS-induced production of inflammatory cytokines, chemokines and mediators and also ameliorate inflamed leucocyte migration, cell metabolic and immune reactions, and thereafter decrease inflammatory response.

The results also showed that both IL-37a and b significantly suppressed the expression of tyrosine-protein phosphatase non-receptor type-6 (PTPN6) or SHP-1, and IL-37b suppressed Phosphatidylinositol-3, 4, 5-trisphosphate 5-phosphatase-1 (Inpp5d) encoding SHIP in LPS-stimulated spleenocytes. Thus, down regulation of SHP-1 and/or SHIP may contribute to IL-37-enhanced antibody production and proliferation in B cells while previously in Chapter 4 were mentioned that IL-37 signals increased IgG production and cellular proliferation in LPS-stimulated murine CD19⁺B cells.

Conclusion and prospects:

My results demonstrated for the first time that both the endogenous and exogenous IL-37a is bioactive with immune regulatory property. IL-37a is highly inducible by inflammatory signals. Intriguingly, while IL-37a and b inhibit inflammatory response in macrophages, both the cytokines increase antibody production in murine and human CD19⁺ B cells. This strongly suggests that IL-37 may not only suppress but also enhance immune response depending on cell types. The success in the production of recombinant IL-37a and b allows further study, and to compare their function in many immunological and biochemical conditions in vitro and in vivo. I found that, while IL-37a and b share the functional similarity, they are different in transcriptomics and some functions; this may be due to their difference in protein sequence, in particularly the NLS in the N-terminal. More works is needed to answer the question. Altogether, IL-37a is a previously unrecognised immunoregulatory cytokine and may play an important part in immune tolerance and the regulation of inflammatory disorders.

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.....

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Author's Declaration

The work presented in this thesis represents original work carried out by the author, with the exception of NGS/microarray/lab work part(Chapter 5) have been done in Dr.Xu lab/ in China. This thesis has not been submitted in any form to any other University. An appropriate acknowledgement made in the text to those who provided me with reagents, materials and technical support.

Signature:

Najwa J Hameed

Abbreviations

Ab - Antibody
 ADCC - Antigen-dependent cell-mediated cytotoxicity
 Ag- Antigen
 AKT - Protein kinase B (PKB)
 AMCDC - Antibody-mediated complement-dependent cytotoxicity
 ANOVA -Analysis of variance
 AP - Activator protein
 APC - Antigen presenting cells
 April - A proliferation-inducing interleukin ligand
 ARG - Arginase
 B cells - B lymphocytes
 BAFF -B-cell-activating factor
 BCR - B-cell receptor
 Blimp-1 -B lymphocyte induced maturation protein-1
 BLNK -B-cell linker protein
 BTK - Bruton's tyrosine kinase
 CC - Cervical cancer
 CCL - Chemokine ligand
 CD - Circular dichroism
 CD - Cluster of differentiation
 CD - Crohn's disease
 Cdk - Cyclin-dependent kinases
 cDNA- complementary deoxyribonucleic acid
 CDSP - Cytotoxic DNA-sensing pathway
 CLP - Common lymphoid progenitor
 CLRs - C-type lectin receptors
 CPG - Cytosine-phosphate-guanine
 CSR - Class switch recombination
 CTL - Cytotoxic T lymphocyte
 CXCL - CXC chemokine ligand
 CXCL2 Chemokine (C-X-C motif) ligand 2
 DAMPs - damage-associated molecular patterns
 DCs -Dendritic Cells

DEG - Differentially expressed genes
DJ - Diversity and joining
DMEM -Dulbecco's Modified Eagle Medium
DPBS - Dulbecco's Phosphate Buffered Saline
E. Coli - *Escherichia coli*
EAE - Experimental autoimmune encephalomyelitis
EBV - Epstein-Barr virus
EDTA - Ethylenediamine tetra-acetic acid
ELISA - Enzyme linked immunosorbent assay
ERK - Extra-cellular signal-regulated kinase
Fab - Fragment antigen binding
FACS - Fluorescence assisted cell sorting
FBS - foetal bovine serum
Fc - fragment crystallisable
FcR - Fragment crystallisable receptor
FCS - foetal calf serum
FLIL - Full-length interleukin
FOXP-3 - Forkhead box proteins-3
FTICR -Fourier transform ion cyclotron resonance
Fyn - Proto-oncogene tyrosine-protein kinase
GAPDH - glyceraldehyde-3-phosphate dehydrogenase
GATA3 - GATA-binding factor
GC - Germinal centre
G-CSF - Granulocyte colony-stimulating factor
GD - Graves' disease
GM-CSF - Granulocyte-macrophage colony-stimulating factor
Grb-2 -Growth factor receptor-bound protein-2
GSK - Glycogen synthase kinase
GSK3 - Glycogen synthase kinase 3
GST - Glutathione S-Transferase
H₂SO₄ - sulphuric acid
HBsAG - Hepatitis B surface antigen
HCC - Hepatocellular carcinoma
HCl - Hydrogen chloride
HDM - house dust mite

HIV - Human immunodeficiency virus
HPV - Human papilloma virus
HRP - Horse radish peroxidase
HSC - Haematopoietic stem cell
IAV - Influenza A virus
IBD - Inflammatory bowel disease
IC - The immune complex
IFN - Interferon
IFN β -1 - interferon-beta 1
IFN γ - Interferon gamma
Ig - Immune globulin
IGF -Insulin growth factor
IHC - Immunohistochemistry
IKK - I κ B kinase
IL - Interleukin
ILCs - Innate lymphoid cells
iNOS - inducible nitric oxide synthase
Inpp5d- Phosphatidyl inositol-3, 4, 5-trisphosphate 5-phosphatase 1
IP3 - Inositol-(1, 4, 5) P3
IPTG - Isopropyl β -D-1-thiogalactopyranoside
IRAK-1 - IL-1R associated kinase1
IRAK-4 - Interleukin receptor-associated kinase
IRF - Interferon response factor
ITAM - Immunoreceptor tyrosine-based activation motif
JAK - Janus kinase
JCSG - Joint Centre for Structural Genomics
JNK - JUN N-terminal kinase
JUN - JUN proto-oncogene
KEGG - Kyoto Encyclopedia of Genes and Genomes
LABA - Long acting β 2-agonists
LAL - Limulus amebocyte lysate
LB - Luria broth
L-chain - Light chain
LPS - Lipopolysaccharide
LRR - Leucine rich repeats

LT - Lymphotoxine
LYN -Src-family kinase
M1 -Classically activated macrophages
M2 - The alternative macrophages two
MACS -Magnetic-activated cell sorting
MAL - Myeloid adaptor-like protein
MALDI - Matrix-assisted laser desorption and ionization
MAPKs - Mitogen-activated protein kinases
MBP - Maltose binding protein
MC -Mast cell
MCP - Monocyte chemoattractant protein
M-CSF -Macrophage colony-stimulating factor
mDC -Myeloid dendritic cell
MEK - MAP/ERK kinase
MEKK-MAP/ERK kinase kinase
MEKKK - MAP/ERK kinase kinase kinase
MHC - Major histocompatibility complex
MHC-I - Major histocompatibility complex one
MHC-II - Major histocompatibility complex two
MIDAS-HT-96 -Modern Intelligent Dynamic Alternative Screen
MIP - Macrophage inflammatory protein
MKK - Map kinase kinase
Morpheus-HT-96 - 3D protein crystallization screen incorporating a range of low molecular weight ligands
MS -Multiple sclerosis
mTOR- Mammalian target of rapamycin
MyD88 -Myeloid differentiation primary response gene (88)
MZ - Marginal zone
NFAT-Nuclear factor of activated T cells
NF- κ B -Nuclear factor kappa-light-chain
Ni - Nickel
Ni-NTA - Nickel nitrilotriacetic acid
NK- cells Natural Killer cells
NLR - Nod like receptor
NLS - Nuclear localisation sequence

NMR - Nuclear magnetic resonance technique
 NO - Nitric oxide
 NOD -Nucleotide-binding oligomerization domain
 SCID -Sever combined imunne deficiency
 Nras - Neuroblastoma RAS vial oncogene homologue
 NSCLC - Non-small cell lung cancer
 OVA - Ovalbumin
 Ox-LDL - Oxygenated low-density lipoprotein
 PACT -PACT premier is a pH, Anion, Cation crystallization trial
 PAMP - Pathogen associated molecular patterns
 PBMCs - Peripheral blood monoclonal cells
 PBS - Phosphate buffered saline
 PCs - Plasma cells
 PH - Potential hydrogen
 PI3- kinase Pphosphoinositide-3-kinase
 PI-3K - Phosphatidylinositol-3 kinase
 PIP-3 - Phosphatidylinositol triphosphate
 PLC - Phospholipase C
 PLTP - Phospholipid transfer protein
 PPARr - peroxisome proliferator-activated receptors
 PRRs- Pattern recognition receptors
 PTKs- Protein tyrosine kinase
 PTPN - Tyrosine-protein phosphatase non-receptor
 PTEN- Phosphatase and tensin homolog
 qPCR - Quantitative polymerase chain reaction
 RA - Rheumatoid arthritis
 RANK - receptor activator of NF-kB
 RANKL -receptor activator of NF-kB ligand
 RAS - Reactive oxygen species
 RAW - Murine macrophage cell line
 RIG-RLRs - Retinoic acid-inducible gene-like receptors
 RING -Really Interesting New Gene
 RIP - Receptor interacting protein
 RNA - ribonucleic acid
 ROS -Reactive oxygen species

RPMI - Roswell Park Memorial Institute
RT-PCR - Reverse transcription polymerase chain reaction
SDS - Sodium dodecyl sulphate
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC - Size exclusion chromatography
SHIP -SH2 domain-containing inositol 5' phosphatase
SHM - Somatic hypermutation
SHP -Src homology region 2 domain-containing phosphatase
SIg - Secretory immune globulin
SIGIRR -Single Ig IL-1-related molecule
siRNA - Small interfering RNA
SLC - Surrogate light chain
SLE - Systemic lupus erythematosus
SP - Streptococcus pneumonia
SRC -Proto-oncogene tyrosine-protein kinase Src
sST- soluble suppression of tumorigenicity 2
ST2 - Suppression of tumorigenicity 2
STAT - Signal transducer and activator of transcription
SYK -Spleen tyrosine kinase
T cells - Thymus lymphocytes
TAB -TGF-beta-activated kinase1 and MAP3K7-binding protein 2
TAK1 -Transforming growth factor beta activated kinase-1
TANK - binding kinase
TBE - Tris-borate-EDTA
TBK - kinase
TCR - T cell receptor
TD - T-cell dependent
tDC - Tolerogenic dendritic cells
Tg -Transgenic
TGF - Transforming growth factor
Th cell - T helper cell
THP-1 - Human monocyte cell line
T-cell ID - T-cell independent antigen
TIR - Toll interleukin resistance
TIRAP -Toll/interleukin-1 receptor adaptor protein

TKB - Tyrosine kinase-binding

TLR - Toll-like receptor

TNF - Tumour Necrosis Factor

TNF α - tumour necrosis factor- α

TRAF - Tumour necrotic factor receptor-associated factors

TRAM - TRIF-related adaptor molecule

Treg - Regulatory T cells

TRIF - TIR-domain-containing adapter-inducing interferon- β

UC - Ulcerative colitis

UCMSCs - Umbilical cord-derived mesenchymal stem cells

Vav - Proto-oncogene vav

V-DJ - Variable diversity and joining

VEGF - vascular endothelial growth factor

VEGF-C - Vascular endothelial growth factor-C

WT - Wild type

The units

-/- Knock out gene deletion

°C Degree Celsius

µg - microgram

µl - microlitres

µM - micromolar

gm - gram

kDa - kilo daltons

M - molar

mg - milligram

ml - millilitres

mM- millimolar

P value (P) Probability value

SD - Standard deviation

UV - Ultraviolet

V - volt

Xg - times gravity

Chapter1

Introduction

1 Introduction

1.1 The Immune System

The human immune system is composed of immune organs, immune cells and effector immune molecules; that work together in a complementary way to protect against infection. The immune response is a process of recognition and elimination of foreign antigens and is divided into innate and adaptive immune response (Abbas et al, 2012). The immune system functions to defend against invading pathogens including viruses, bacteria, parasites and fungi. Moreover, the immune system can also detect and eliminate necrotic, apoptotic and transformed cells such as tumour cells caused by gene mutagenesis (Delves and Roitt, 2011). Although the immune system protects the body against pathogens, dysfunction of immune balance can cause certain diseases (Murray et al, 2012).

1.1.1 Innate immunity

The innate immunity is the first line of defence against pathogens via tissue barrier and innate immune cells. It is unspecific, rapid, without immunological memory and controls the infection quickly within the first few days using several strategies, including pro-inflammatory signalling cascades activation, opsonisation, phagocytosis and activation of complements (Abbas et al, 2012).

The innate immunity detects and recognizes microbial invaders through innate immune cells via their pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), that recognize pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) to trigger immune response (Armant and Fenton, 2002; Abbas et al, 2012). Following pathogen recognition, multiple immune defence mechanisms and pathways are activated including phagocytosis and killing by phagocytic cells; complement pathway activation; acute inflammatory responses activation by cytokines and chemokines; transcription of several anti-viral proteins by type I IFN that leads to viral infection clearance. Therefore, pathogens recognition via PRRs/TLRs activates several acute immune responses that are necessary to limit the early spread of infection, while a sophisticated tool of specificity and memory to re-infections countering is provided by activation of adaptive immune responses

(Pasare and Medzhitov, 2001). The innate immune system can also activate and shape the adaptive immune response through cytokines, antigen presentation and co-stimulatory molecules (Pasare and Medzhitov, 2005).

1.1.1.1 Tissue barrier

Tissue barriers are the first line of defence to prevent infection; these include:

1. Mechanical barriers that restricts pathogen entrance such as skin epithelial cells that are joined by tight junctions in the skin, gut, eyes, lungs and nose.
2. Chemical barriers involving secreted chemical molecules that can destroy pathogens in different ways such as stomach pepsin enzyme and low pH in the gut, lactic acid from sweat glands, lysozyme in the saliva.
3. Microbiological barrier including normal flora and microbiotas (Janeway et al, 2007).

1.1.1.2 Innate immune cells

Innate immune cells are developed from myeloid progenitors in the bone marrow, the key innate immune cells including macrophages, neutrophils, and dendritic cells. With one exception, innate lymphoid cells (ILCs) that are developed from lymphoid progenitors are the same as T and B cells but without antigen receptors (Abbas et al, 2012).

1.1.1.2.1 Macrophages

Macrophages are generated from monocytes and can differentiate into tissue specific macrophages such as Kupffer cells, alveolar macrophages, and microglia in different organs. The main function of macrophages is their critical role as phagocytic immune cells in the host defence against infectious antigens, cleaning the cell debris and apoptotic cells (Aderem and Underhill, 1999; Alber et al, 2012). Macrophages are also involved in wound healing and immune regulation. Macrophages are one of the professional antigen presenting cells (APCs) and can initiate T cell-mediated adaptive immune response; thus

macrophage can be a key linker between the innate and adaptive immunity (Yurasov et al, 2005).

According to their function, macrophages can be differentiated into two activated subtypes: classically activated macrophages (M1) and alternative macrophages (M2) in response to different activation conditions. M1 is polarised in response to (LPS), IFN γ and IL-12. M1 macrophages express pro-inflammatory cytokines and inducible nitric oxide synthetase (iNOS), an enzyme for the production of nitric oxide (NO) which has a major role in killing microbes through a respiratory burst mechanism (Babior et al, 1984). These classic macrophages resist microbial infection through NO and IFN γ that mediate the antimicrobial and tumoricidal properties of M1 macrophage. The dysregulation of M1 may also lead to inflammatory diseases (Parisi et al, 2018). M2 Macrophages or alternatively activated M2-macrophages are generated in the presence of IL-13 and IL-4, which release IL-13 and transforming growth factor beta (TGF- β), and express prototypical marker arginase (Hesse et al, 2001). The M2 macrophages contribute to wound healing and tissue repair (Levings and Schrader, 1999) and protection against parasite infection. However, over-activation of M2 cells may cause tissue remodelling, fibrosis and allergic conditions.

1.1.1.2.2 Neutrophils

Neutrophils are classified as granulocytes due to the abundance of granules in their cytoplasm. They are the most abundant leucocytes, comprising 60-70% of white blood cells (Abbas et al, 2012). Neutrophils are the first cells recruited to the site of infection, and are essential for immune defence against bacteria and fungi. Neutrophils are phagocytic cells and can kill pathogens by the toxic chemicals and enzymes in their granules, including defensins, eosinophil cationic protein, lysozyme and superoxide (Segal, 2005). However, neutrophils are short lived, with survival about 4-5 days in the peripheral blood. Thus, they are mainly involved in the early immune activation against infection and inflammation.

1.1.1.2.1 Dendritic cells

Dendritic cells (DCs) are also generated from monocytes. DCs localise in the tissues and there are three types of DCs that have been identified: conventional dendritic cells, plasma cystoid DCs and the myeloid dendritic cells, which are

derived from blood monocytes (Plantinga et al, 2010; Spears et al, 2011). While DCs are located in the tissues, their distinct morphology enables them to move to lymph organs all over the body. The main function of DC is to act as professional APC. DCs can process antigen, upload it onto their surface major histocompatibility complex (MHC-I) and (MHC-II) molecules and present the peptide antigen to T cells. As a professional APCs, DCs can not only provide the antigen signal to naive T cells, but also the co-stimulatory signals via CD80/86 and CD40L as well as cytokine signals (Abbas et al, 2012). The three signals lead to the activation and proliferation of T cells and the differentiation of naive T cells into effector T cells (Ni and O'Neill, 1997). Thus, DCs are considered to be the key bridging cells between innate and adaptive immunity, in addition, they also generate reactive oxygen species (ROS), NO, and anti-microbial proteins that kill intracellular pathogens.

1.1.1.2.2 Natural killer (NK) cells

NK cells are lymphocytes derived from common lymphoid progenitor, the same as B cells and T cells are derived. However, NKs do not express antigen-binding receptors as B and T cell. They are cytotoxic cells and kill tumour and virus-infected cells. NK cells kill other cells via secretion of cytotoxic molecules, such as perforin and granzyme proteases that induce apoptosis in the target cells. NK cells can also kill the target cells via other pathways, including antibody-dependent cell-mediated cytotoxicity (ADCC) and cytokine-induced NK and cytotoxic T lymphocyte (CTL) activation (Abbas et al, 2012).

1.1.1.2.3 Innate lymphoid cells (ILCs)

ILCs are innate immune cells, which are derived from common lymphoid, but not myeloid progenitors. They are lymphocytes, but different from T and B cells they do not express antigen receptors. They are mainly located in the tissues and are innate immune cell lineages (Buonocore et al, 2010; Moro et al, 2010). Functionally, they are associated with the innate immune response. However, ILC1 and ILC3 can also express MHC II and can serve as APC. Based on cytokine production and transcription factors, ILCs can be classified into three subsets (Spits et al, 2013; Walker et al, 2013):

- ILC1 cells express transcription factor T-bet and produce IFN- γ upon stimulation by IL-12 and IL-18. ILC1 cells shares functional similarity with Th1 cells and plays important role in protection against intracellular infection. The over-activation of ILC1 may lead to inflammatory disorders (Gordon et al, 2012).
- ILC2 cells express GATA3 and produce IL-5, IL-9 and IL-13 cytokines in response to IL-25 and IL33. Therefore, ILC2 cells are closely associated with Th2 cells and play critical role against parasite infection. Dysregulation of ILC2 cells can cause allergy and asthma by overproduction of type II cytokines (Hoyler et al, 2012).
- ILC3 cells express ROR γ t and produce Th17 cytokines IL-17a and IL-22 in response to IL-23 and IL-18. ILC3 cells are important in defence against extracellular pathogens. However, abnormality of ILC3 may lead to autoimmune disorders, the same as Th17 cells (Spits et al, 2013).

1.1.2 Adaptive immunity

The adaptive immune system consists of T and B-lymphocytes cells. These cells express surface antigen receptors. The adaptive immune response includes humoral response induced by B cell and cell mediated response by T cell (Murphy, 2011). The adaptive immune response appears later than innate immune response and is characterised by antigen specificity and memory development (Sattler et al, 2013).

Immunological memory is crucial for the strong and specific response in the host defence against reinfections (Kindt et al, 2007; Abbas et al, 2012). The adaptive immune response develops through four phases:

1. Initiation: it is called the priming phase in that the T and B cells recognise antigen through their antigen receptors; T cells via their T-cell receptor (TCR) and B cells via B-cell receptor (BCR).

2. Activation and differentiation phase: lymphocytes that recognise antigens and receive co-stimulation and cytokine signals from APCs start the proliferation and differentiation into effector cells.
3. Effector-stage: T and B effector cells eliminate pathogens via their related effector molecules.
4. Recovery phase: some effector cells develop into memory cells against reinfection and most of them are eliminated by apoptosis (Murphy, 2011).

1.1.2.1 T Cell-mediated immunity

T cells (CD4⁺ and CD8⁺ T cells) originate from bone marrow common lymphoid progenitors and develop into mature T lymphocytes in the thymus. The mature antigen-naïve T cells will differentiate into effector cells in the peripheral lymph organs when they sense antigen from APCs.

Effector T cells lead the cellular immune response against microbial infection. The defence mechanism that both CD4⁺ and CD8⁺ T cells provide is via their cell surface receptors and secreted molecules, mainly the cytokines and chemokines (Delves et al, 2011).

The cellular immune response is initiated by antigen-presenting cells (APC), which could be macrophages, B cells or the DCs. DCs are the most efficient antigen-presenting cell type. To induce optimal activation and differentiation of naïve into effector T cells, APCs need to provide three signals to naïve T cells (Garcia and Adams, 2005): signal 1 is initiated when T cells through their T cell receptor (TCR) recognise the processed peptide antigen presented by APC via the MHC molecule. The second signal is induced by the interaction of ligand/receptor pairs of co-stimulatory molecules on both APCs and T cells, including CD80 on APC and CD28 on T cells. While the two signals can result in the activation and proliferation of T cells, these are not enough to induce the differentiation of naïve T cells into effective T cells if the cytokine signals are not provided. Once the three signals are received, naïve CD8⁺ T cells differentiate into cytotoxic T cells which will kill infected cells and tumours (O'Shea and Paul, 2010), while naïve CD4⁺ T cells differentiate into several

subsets including T helper (Th) 1, Th2, Th9, Th17 and T-regulatory cells (T-reg) (O'Shea and Paul, 2010). This differentiation depends on the milieu of polarisation cytokines, for example, IL-12 is required for Th1 polarisation and IL-4 for Th2 development (Fig 1.1). Th1 cells play an important role in the protection against intracellular pathogen infection and in inflammatory diseases if over-activated; Th2 cell differentiation is associated with anti-parasite infection. However, dysregulation of Th2 responses may also cause allergy and asthma. Th17 cells are closely associated with anti-fungal and extracellular bacterial infection. Abnormality of Th17 response may lead to inflammatory and autoimmune diseases (Weaver et al, 2013). The T-reg cells are the suppressive T cells in immunity and disease and play an important role in immune balance and tolerance. Effector T cells have short life spans, however some of them can differentiate into memory T cells which are long-lived and expand quickly upon cognate antigen re-exposure. Memory cells are important in protection against reinfection (Sprent and Surh, 2002; Garcia and Adams, 2005; O'Shea and Paul, 2010).

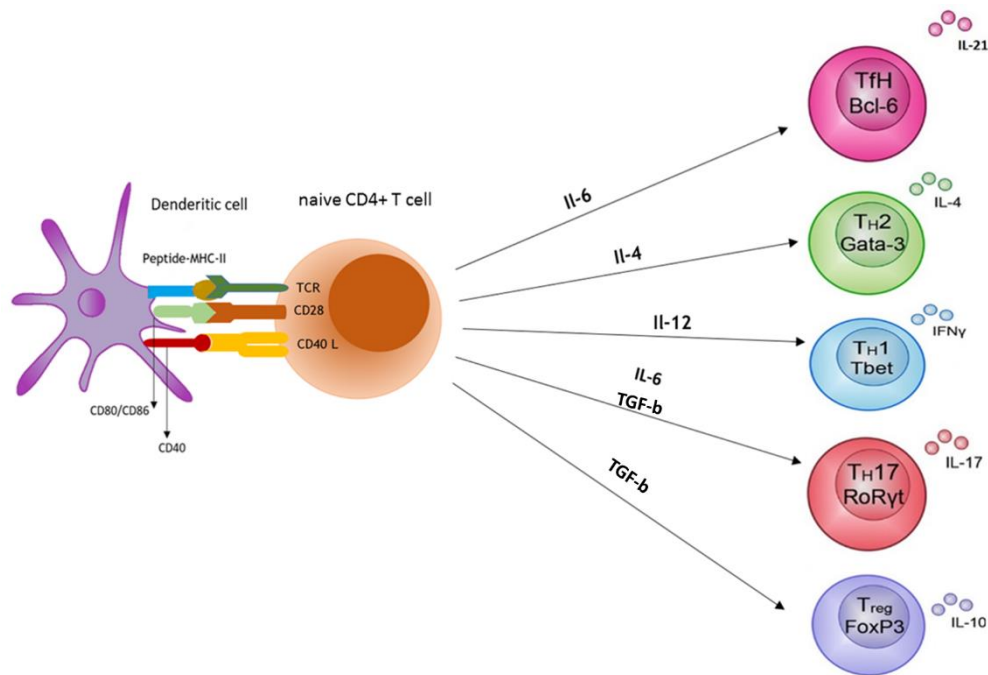


Figure 1.1 CD4+ T cell subsets.

Upon encountering with foreign antigens and costimulatory molecules presented by DCs, naive CD4+ T cells differentiate into TfH, TH1, TH2, TH17 and Treg cells. These differentiations are shaped by cytokines, and are characterized by the expression of lineage-specific transcription factors and the production of signature cytokines. In response to IL-6, naive CD4+ T cells differentiate into TfH cells which is important for germinal center B cell; IL-4 is important for the differentiation of TH2 cells that play a crucial role in antiparasite; and humoral immunity. IL-12 is required for the differentiation of TH1 cells. IL-6 together with TGF- β drive naive T cells to develop into TH17, while TGF- β alone promotes naive T cells to differentiate into Foxp3-expressing Treg cells. TCR (T cell receptor); CD40 L (CD40 ligand); Gata-3 (Gata-binding factor); B-cell lymphoma 6 protein; T-bet (T-box transcription factor); ROR γ t (RAR-related orphan receptor gamma t) FOXP3 (forkhead box P3).

(Modified from O'Shea, J. J. & Paul, W. E. (2010). Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science*, 327, 1098-1102.)

1.1.2.2 B cell-mediated humoral immunity

B cells and the antibodies they produce drive the humoral immune response; these antibodies protect the body against extracellular pathogens. B cells activation and differentiation into plasma cells that produce antibodies depend on antigen and Th cells. T-cell independent (ID) antigens do not require T cell help, but T-cell dependent (TD) antigens require Th cells. The B cells in the periphery are present as B1 and B-2 cells subset, and they can be distinguished by their surface markers, origin, location, and distinct antibody production profile (Fig 1.2) (Fagarasan, 2000; Martin and Kearney, 2001).

B cells bind to antigens through somatically mutated specific receptor called B cell receptor (BCR), which triggers the first signal for B cell activation. B cells can be activated by other stimuli, such as the CD40 ligand on T cells interact with CD40 on B cells, and IL-4, IL-5 and IL-13 cytokines secreted by T cells. Furthermore, B cells express TLRs that in response to their ligand provide an additional signal for B cell activation (O'Rourke et al, 1997). Upon activation, mature B cells proliferate and differentiate into antibody-producing plasma cells and memory B cells which do not produce antibody, but they can be activated quickly in a recall to the cognate antigen or TLR agonists and rapidly produce a large amount of antibody (Liu et al, 1991; Hardy and Hayakawa, 2001).

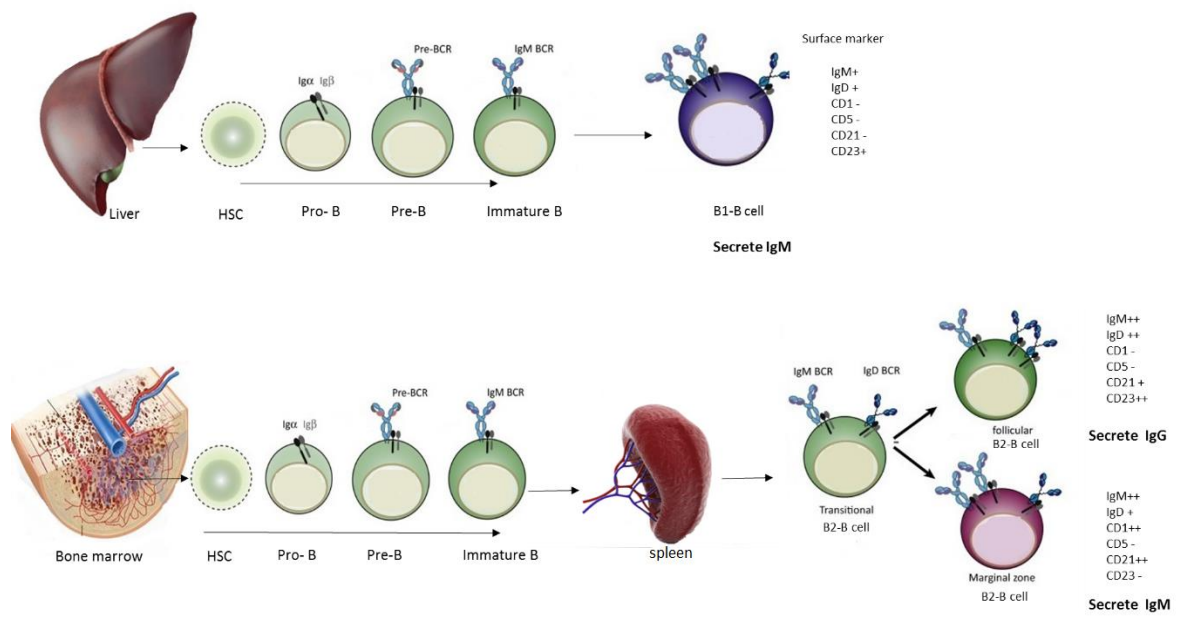


Figure 1.2 B cells subsets

(Modified from Abbas, A, Lichtman, A and Pillai, S. (2012). Cellular and molecular immunology. Philadelphia: Elsevier).

1.2 B cell

1.2.1 B cell development

The same as T cells, B cells are developed from the common lymphoid progenitors (CLP). In human, B cells develop in the yolk sac fetal liver before birth; however, after birth the development is restricted to the bone marrow. While the detailed mechanism is still missing, B cells develop into mature B cells in bone marrow via pro, pre and immature B cell stages (Namen et al, 1988; Svensson et al, 1998). At pro-B cell stage, the rearrangement of the Ig heavy-chain D-J and V-DJ genes occurs. This leads to the surface expression of Ig α and Ig β heterodimers and the developing into pre-B cells (Abbas et al, 2012). Pre-B cells express the pre-BCR consisting of the μ heavy-chain paired with germline encoded $\lambda 5$ and Vpre-B chains; this is called the surrogate light chain that is associated with Ig α /Ig β heterodimers to form the functional pre-B cell receptor that has ability to transduce signal (Schlissel and Baltimore, 1989; Meffre and Wardemann, 2008). Pre-B cell receptor signalling is essential for pre-B cell progression along its developmental pathway.

The pre-BCR generation enables the pre-B cells to further develop into immature B cells followed by the rearrangement of the Ig light-chain locus that results in antigen specificity and increases the B cell repertoire diversity (Schlissel and Baltimore, 1989; Nishimoto et al, 1991; Lassoued, 1993). The successful recombination and BCR expression on the surface of immature B cells allow the cells to recognise specific antigen and the further development by negative selection. During the BCR development, some auto-reactive B cells can be generated due to the BCRs being generated randomly and these cells may cause autoimmune disease if not depleted. To do so, the immature B cells in the bone marrow undergo negative selection, in which they are exposed to self-antigen and the autoreactive cells will be depleted by either receptor editing or clonal deletion. The B cells that are not for self-antigen will escape the selection, become mature B cells and migrate into the periphery tissues. In the periphery, the newly generated B cells or antigen naive B cells are non-dividing cells, with a very short life span, and the majority of them die by apoptosis in the absence of antigen within a few weeks. However, upon antigen recognition, these B cells enter the secondary lymphoid organs such as the spleen in which they survive

and differentiate into effector, plasma and memory B cells (Lam and Rajewsky, 1997; Harless et al, 2001).

1.2.2 B cell subsets

B cell subsets develop from different progenitors. There are two subsets of B cells: B-1 B cells and B-2 B cells. B-1 B cells (CD5+) comprise a minor population about 5% of B-cells (Nishimura et al, 1992). B -1 B-cells are part of innate immunity as their response to pathogen is rapid. B-1 B cells develop from fetal liver haematopoietic stem cells during haematopoiesis, and mainly locate to peritoneal and mucosal cavities (Kantor, 1993). B-1 B cells express surface markers, CD19 and CD54, although CD45R is expressed at low levels in comparison to other B cell lineage; B-1 cells also express CD11, IgM (high), IgD (low) (Rothstein and Kolber, 1998; Abbas et al, 2012).

B-1 cells provide protection in innate immune manner through secretion of natural IgM, for instance in streptococcus infection (Baumgarth et al, 2005). B-1cells promote long-lasting protective immunity to T-cells ID antigens by secreting IgM against pathogenic antigens (Alugupalli et al, 2004). B-1 B cells express low level of BCR, therefore they do not respond properly to activation through BCR (Morris, 1993; Bikah et al, 1996; Rothstein and Kolber, 1998).

B-2 (CD5-) B cells comprise the major population among the other B cell types and are crucial for humoral response to infection. These cells produce high affinity antibodies (IgG, IgE) and can undergo somatic hyper-mutation (SHM), and class switch recombination (CSR). B-2 B cells, in addition, reside in the follicles and can migrate from one to another of the lymph nodes (Abbas et al, 2012).

1.2.3 B cell function

Current evidence suggests that B cell functions are not restricted to antibody production in immune response (O'Neill et al, 2005; Cariappa et al, 2007). Effector B cells have three main functions: cytokines secretion, serving as APC, producing antibody:

1.2.3.1 Cytokines secretion

B Cells contribute to and modulate immune response through secretion of a wide range of cytokines that influence the outcome of the immune response. B cells secrete different types of cytokines, including inflammatory and regulatory cytokines, thus B cells can regulate all types of immune response; for instance, B cells secrete Th1 and Th2 cytokine IFN γ and IL-4 and can affect Th1 cells and Th2 cell development and functions, respectively (Harris et al, 2005a; 2005b). Effector B cells also produce IL-6 and other pro-inflammatory cytokines such as IL-12, TNF α , and lympho-toxins, which are important in shaping secondary lymphoid organs (Lund, 2008). In addition, these pro-inflammatory cytokines produced by B cells can contribute the severity of autoimmune disease. In Experimental autoimmune encephalomyelitis (EAE), specific B cells producing IL-6 are associated with the EAE disease the severity (Barr et al, 2012). However, in another study, B cell deficient mice in EAE models revealed an increase in the disease severity; suggesting that these cells are necessary to regulate the disease progression (Wolf et al, 1996). B cells modulate immune response through producing IL-10, which inhibits inflammatory cytokine secretion from monocytes and macrophages (Fiorentino et al, 1991). Furthermore, B cells can also regulate immune responses by the induction of regulatory T cells via their secretion of IL-10 and TGF- β (Akdis and Blaser, 2001; Fillatreau et al, 2002; Matsushita et al, 2010).

1.2.3.2 Antigen presenting cells (APC)

B cells are efficient antigen presenting cells, due to their ability to endocytosis antigen via BCR and present it via surface MHC-II and I molecules. BCR is able to concentrate small amount of antigens and present it efficiently to Th cells, which are resident in the secondary lymphoid organs together with B cells (Lanzavecchia, 1985; Vanderveen et al, 1992). In addition, B cells can also provide costimulation and cytokine signals. B cell APC are important in the development of autoimmune diseases.

1.2.3.3 Antibody production

B cell defence against pathogens is mainly carried out through their specific antibody production. It has been shown that antibody deficiency results in

susceptibility to infection (Duraisingham et al, 2014). Although antibodies do not directly remove pathogens, they can do so indirectly, via binding to antigens and a broad mediating range of effector responses (Lanzavecchia, 1985; Vanderveen et al, 1992).

1.2.4 Antibodies

Antibodies are immunoglobulins which are a heterodimeric protein composed of two heavy (H) and two light (L) chains. Functionally, an antibody has a variable domain (V) in Fab part, which binds to antigens and an FC part in constant H chain domains (C). The variable domains are subjected to SHM after exposure to antigen, which increases the antibody specificity to antigens (Arnold et al, 2007). The FC part has specific effector functions involved in complement activation (IgG and IgM) and Fc receptor binding.

1.2.4.1 Antibody types

Based on the difference in heavy chain sequence, the antibodies have five classes: IgM, IgG, IgA, IgD, and IgE isotypes.

1.2.4.1.1 IgM

IgM is the biggest immunoglobulin with five protein units. IgM is expressed on naive B cells surface and associated with both CD79a and CD79b. IgM is the first immunoglobulin expressed during B cell development, and is a pentameric immunoglobulin with high avidity and low affinity towards antigens. IgM has short lifespan of about 3 days and is associated with both innate and adaptive immune response, in which it has ability to activate complement through opsonizing or coating the target antigen (Boes, 2000).

1.2.4.1.2 IgD

The IgD immunoglobulin has very low levels in the serum, and it is a monomer immunoglobulin with a short life span of two days. The IgD expressed on mature B cell surface as bounded immunoglobulin associated with Ig α and Ig β for signalling (Geisberger et al, 2006; Riesbeck and Nordstrom, 2006).

1.2.4.1.3 IgG

The IgG is a monomer with four subclasses, IgG1; IgG2; IgG3; and IgG4 (Schroeder and Cavacini, 2010). IgG is the predominant immunoglobulin isotype among other immunoglobulin isotypes in the body with the longest life span of 21-28 days. IgG mediates the clearance of pathogens through opsonisation and complement cascade activation, and all the subclasses can trigger complement fixation with exception of IgG4 that fails to fix complement. IgG subclasses affinity for the first component of the complement (C1q) differs with (IgG3>IgG1>IgG2). In addition, IgG1 and IgG3 bind to all three FcγR classes (FcγR I, II and III), while IgG4 only binds to FcγRII and III (Parlowsky et al, 2003; Kawasaki et al, 2006).

1.2.4.1.4 IgA

The IgA has predominant levels in secretions including saliva and breast milk, and at the mucosal surfaces (Woof and Mestecky, 2005; Schroeder and Cavacini, 2010). IgA is a monomer in the serum with level lower than IgG. Secretory IgA (sIgA) is a dimer and sometimes trimer or tetramer that is associated with a J-chain and has a critical role to protect mucosal surfaces from virus, toxins, and bacteria. Neutrophils express IgA receptor that is important for local clearance of pathogens through antibody dependent cell cytotoxicity (ADCC) (Stubbe et al, 2000).

1.2.4.1.5 IgE

The IgE has the lowest serum concentration with the shortest life span. While IgE is closely associated with hypersensitivity and allergic reactions, its main functions protective against parasites and worm infections. The IgE has high affinity to the FcεRI receptor, which is expressed on basophils, eosinophils, mast cells and Langerhans cells (Chang et al, 2007).

1.2.4.2 Antibody function

1.2.4.2.1 Phagocytosis

Antibody is one of the phagocytosis receptors on the surface of phagocytes and can trigger phagocytosis process. Antibody can fix pathogens on the surface of phagocytes, including monocyte, macrophage and neutrophils. When an antibody

binds to a pathogen with its Fab fragment, its Fc fragment will bind to the Fc receptor on the surface of the phagocytic cells. Following successful engagement, the phagocytes will engulf the targeted pathogen or cells and destroy it (Nimmerjahn and Ravetch, 2007; 2008).

1.2.4.2.2 Neutralization

Some antibodies can bind to antigen and block the function or bioactivity of the antigen. For instance, anti-IL-1 neutralising antibody can bind to and block IL-1's ability of binding to its receptor (Botsios, 2005); also, some antibodies against pathogen surface proteins can prevent the pathogen to infect host cells via the antigen's receptor on host cells (Hessell et al, 2007; Forthal, 2014). Neutralization is a key function of antibody that depends on antibodies' capability without the help of effector cells or other molecules.

1.2.4.2.3 Complement activation

IgM and IgG can kill pathogen, tumour or pathogenic cells via antibody-mediated, complement-dependent cytotoxicity (AMCDC). This is an immune killing mechanism via the classic complement activation pathway. When the antibodies bind to their surface antigen on pathogen or pathogenic cells via their Fab fraction to form an immune complex, this will trigger the recruitment and activation of complement protein (C1) which binds with its C1q subunits through binding to the Fc portion of IgM or IgG. The activation of C1 will activate downstream complement activation cascades to form the membrane attack complex on the surface of pathogen and kill the pathogen by osmotic lysis of the cells. (Roitt et al, 2001; Abbas and Lichtman, 2012).

1.2.4.2.4 Antibody dependent cell cytotoxicity (ADCC)

ADCC is an independent mechanism from the complement system. It is part of the adaptive immune response since the antibody has major role in this biological process. The IgG coats the antigen on target cells via Fab domain, its Fc part can also bind to NK cell FC receptors FcγRIII (CD16) receptor on their surface. This cross-linking will lead to the activation and degranulation of NK cells to release the toxic cytoplasmic granules containing perforin, granzymes, and cell death by apoptosis (Wallace et al, 1994; Horner et al, 2007; Chen and Freedman, 2008; Tudor and Bomsel, 2011).

1.2.5 B cell function in immunity and disease

1.2.5.1 B cell response to T- independent and T-dependent antigens

Based on the requirement for T cell help in B cell activation, B cell responses to antigens can be classified into TD antigen response (TD-antigen) and T-cells ID antigen response (TI-antigen). TD-response is induced by TD antigen and mainly mediated by B-2 cells with immune memory, while TI-response is induced by TI antigen and mainly associated with B1 cells IgM production and without immune memory.

Majority antigens are the TD antigens, including pathogen and foreign proteins that activate B cells via BCR with the requirement of direct help with T-cells. The process occurs in the secondary lymphoid follicles. In order to obtain T cell help, B cells present the antigen on the MHC II molecule to Th cells. After antigen recognition, T cell provide help to B cells through CD40L on that interaction with CD40 R on B cells. This interaction initiates B cell activation and drives B cells into the cell cycle. Together also with the cytokine milieu secreted by T cell at the time of activation, all these supporting signals lead B cell clonal expansion and become effector cells and plasma cells that are able to produce antibodies and cytokines. The plasma cells that undergo further SHM for their BCR affinity maturation and antibody class switch resulting in various antibody isotypes that are more functionally versatile and enhance the humoral response (Parker, 1993; Haas et al, 2011).

In contrast, B cell T-cells ID antigen response does not require T cells help. While follicular B-2 cells can mediate TD-antigen response, TI-antigens; are mainly mediated by marginal zone and B1 cells, and results mainly in IgM secretion (Abbas et al, 2012).

TI-antigens fall into two types with different acting mechanisms: TI- type I antigens are mitogenic stimuli or polyclonal B cell stimulants with intrinsic B cell activating activity that directly drive the B lymphocyte proliferation and differentiation. This activation can be mediated with or without BCR stimulation, for example, LPS and CPG activate B-cells via Toll-like receptors.

This type of B cell immune response is rapidly activated, and does not require T cell help; thus, it is important in protection against extracellular pathogens at early stages of infection (Haniuda et al, 2011; Grant et al, 2012).

TI- type II antigens are mainly the polysaccharide antigens, for instance, the highly repetitive surface epitopes of encapsulated bacteria can activate mature B-lymphocytes. TI-type II antigen activate B cells through cross-linking of BCR with the antigens. Cross activation of these receptors results in proliferation, differentiation, and antibodies production in B-cells (Obukhanych et al, 2006).

A subset of B cells that contributes to immunological tolerance are called regulatory B cells (B-Reg). This type of B cells is immune suppressive by releasing anti-inflammatory cytokines including TGF- β , IL-10, and IL-35. Expression of IL-10 is a critical feature of B Reg cells in both human and mice; and B Reg cells inhibit effector lymphocytes such as T-cells and B cells as well (Rudensky, 2011). While the transcription factor and detailed development pathways are still unclear, B-reg appear to have multiple subsets and can arise from B cell subset in response to inflammation, including PAMPs, but are not found in normal tissues (Rosser and Mauri, 2015).

1.2.5.2 B cell function in autoimmune disease

B cells have a vital role in pathogen clearance through humoral response; however, balance disruption of B cell function and self-tolerance break down can cause autoimmunity (Samuels et al, 2005; Yurasov et al, 2005). B cells play a central role in autoimmune disease by autoantibodies, pro-inflammatory cytokine production and autoantigens presentation to T cells.

Most of the autoantibodies in autoimmune disorders are IgG isotype that have undergone SHM with T cell help. In autoimmune disease, autoantibodies are considered the preferred marker for the diagnosis and prediction in some autoimmune disorders. These autoantibodies are associated with pathogenic effects by different mechanisms. The autoantibodies may bind to free self-antigen to form immune complexes (ICs), the ICs can circulate in the peripheral tissues and the deposition of these ICs in tissues may trigger complement-mediated cytotoxicity and tissue damage (Rojko et al, 2014). Autoantibodies

acting against endocrine glands may affect organ function through stimulation or inhibition of organ bioactivity (Howard et al, 1987; Michalek et al, 2009; Susuki et al, 2012). For example, in medical conditions such as Gravis disease (hyperthyroidism), autoantibody acting against thyroid can stimulate thyroid growth and produce more thyroid hormone.

Dysregulated profiles of cytokine production by B cells are often seen from patients with autoimmunity, with enhanced production of pro-inflammatory cytokines and diminished anti-inflammatory cytokine secretion (Takai, 2002; 2005). B cells secrete pro-inflammatory cytokines such as IL-6, TNF- α , IFN γ , IL-4, which in turn activate macrophages, modulate DCs migration; regulate T cell functions; and provide stimulatory feedback signal for B cell activation (Lanzavecchia, 1985; Amigorena and Bonnerot, 1999). In autoimmune disease, B cells develop ectopic germinal centres as consequence of chronic inflammation associated with autoimmune disease. Due to the lymph-toxins secreted by T and B cells and infiltrate to the site of chronic inflammation, LT α 1 and B2 promote the resident stromal cells to differentiate into dendritic cells, thus promoting disease progression (Schalke et al, 1985; Nielsen, et al, 2001).

Auto-reactive B cells process and present self-antigens to T cells in autoimmune conditions. B cells' function as APC is clearly seen in autoimmune disease; B cells can potentiate disease by promoting pro-inflammatory T cell responses to the self- antigen (Lehmann-Horn et al, 2013). For example, B cell deficient mice impaired T cell priming and proliferation in a model of autoimmune disorder, suggesting that T cell activation requires B cell antigen presentation (Svensson et al, 1998; Lin et al, 2015).

1.2.6 B cell signalling

The B cells responded to antigens through signalling transduction via BCR complex. BCR complex is consists of BCR and two heterodimer subunits of Ig α (CD79a) and Ig β (CD79b). BCR on B cell surface is for antigens binding (Fig 1.3). BCR intracellular signalling is carried out by the two-heterodimer tyrosine residues CD79a and CD79b that contain immune receptor tyrosine-based activation motifs (ITAMs) (Cambier, 1995). ITAMs are responsible for the initiation and propagation of BCR signalling. Signalling through BCR is triggered

once an antigen binds to BCR, leading to BCR aggregation, followed by activation of the intracellular protein kinases, such as Lyn, a protein tyrosine kinase (PTKs) that is already associated with BCR in an inactive form. Lyn activation triggers ITAM phosphorylation, which in turn enables ITAM to bind more protein kinases blnk, Grb2, SYK, BTK, PLC γ , and Vav.

The BCR signal can be further amplified by CD19, a B cell co-receptor that is cluster with aggregated BCR. CD19 can also be phosphorylated by Lyn and become activated. This leads to direct activation of phosphatidylinositol-3 kinase (PI-3K) and AKT. The PI3K- AKT pathway will lead to the activation of transcription factor NF- κ B. BTK activation via PI3K-PIP3 will activate MAPK and nuclear factor of activated T cells (NFAT) pathways (Tsubata, 1999; Dalporto, 2004).

These activated transcription factors, including NF- κ B, NFAT, MYC, ATF2 and JUN in B cells; will subsequently translocate into cell nucleus, bind to the specific binding site on many genes and results in the expression of related genes (DeFranco et al, 1995; Campbell, 1999). Together, a successful BCR signalling in responding to an antigen will lead to B cell survival, proliferation, migration, and antibody production (Burger, 2011; Choi and Kipps, 2012; Burger and Chiorazzi, 2013).

Antigen-independent pathways can also activate B cells, for instance TLR signalling (Abbas et al, 2012). This will be described in related sections in the introduction.

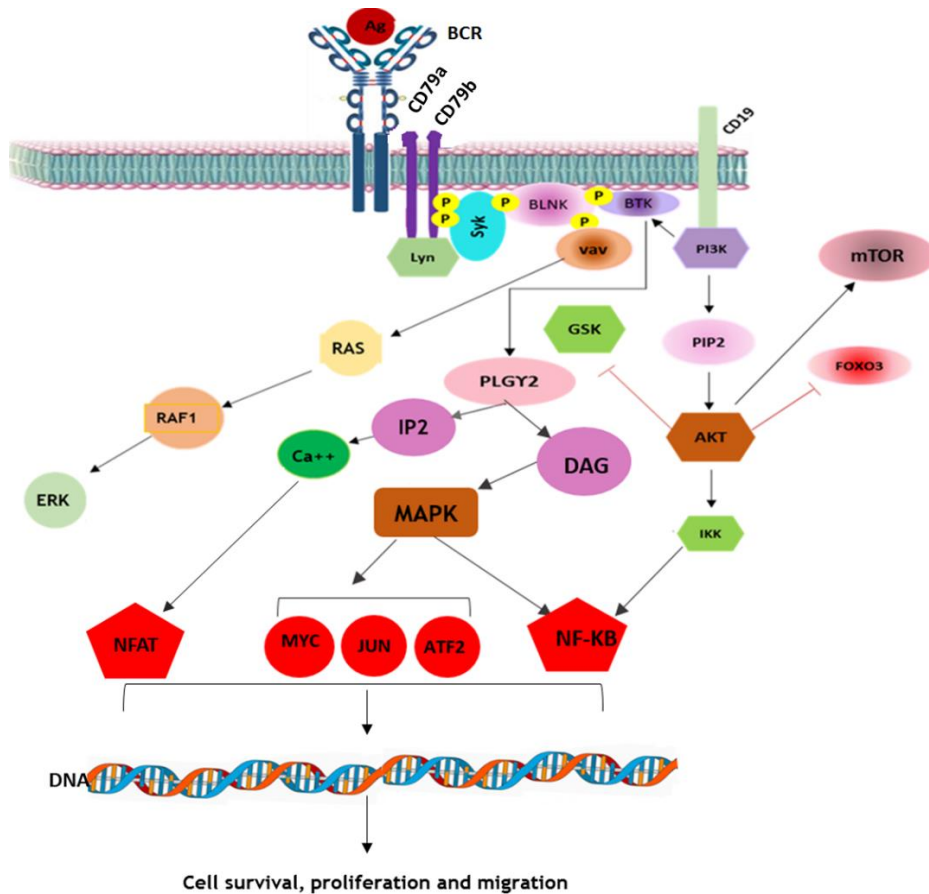


Figure 1.3 B-cell receptor signalling pathway

BCR signaling is composed of a surface Ig molecule associated non-covalently with CD79a and b. Aggregation of the BCR quickly activates Lyn, which activates Syk, and this latter serves as a nucleation site for Bln, Btk, and Vav. These molecules trigger a series of downstream signalling events leading to coordinated gene expression, cell survival, proliferation, and migration. Lyn (Src-family kinase), Syk (spleen associated tyrosine kinase), Bln (B cell linker), Btk (Bruton tyrosine kinase), Vav (guanine nucleotide exchange factor).

(Modified from Choi, M. and Kipps, T. (2012). Inhibitors of B-Cell Receptor Signalling for Patients with B-Cell Malignancies. *The Cancer Journal*, 18(5), pp.404-410).

1.3 Toll-like receptors

Sensing pathogens and tissue damage by innate immune cells is the first step in immune activation against infection. This is attributed to the evolutionary developed the PRRs on/in immune cells. PRRs sense PAMPs and host damage-associated molecular patterns (DAMPs), which play initial role in immune activation. Four groups of PRRs have been identified: TLRs, Retinoid acid-inducible gene (RIG)-like receptors or (RLRs), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs) (Abbass et al, 2012). These PRRs form a network for the detection of all pathogens in or out of cells. The TLRs are the most important group of pattern recognition receptors.

The TLRs are transmembrane or cytoplasmic signalling receptor proteins and belong to the IL-1R/TLR superfamily. Human TLR10 and murine TLR13 have been identified and can discriminate diverse classes of microbial products (Armant and Fenton, 2002; Kaisho and Akira, 2006). TLRs 1-10 are found in human and mice; while TLRs 11-13 are only found in mice not in humans (Takeda, 2004; Kaisho and Akira, 2006). TLRs are widely expressed on immune cells, including macrophages, dendritic cells, mast cells, eosinophils, neutrophils, B and T lymphocytes and tissue cells such as epithelial cells, endothelial cells cardiomyocytes and adipocytes.

1.3.1 TLR cellular location and the recognition of PAMPs

The TLRs are transmembrane proteins characterised by containing three domains; the extracellular domain composed of leucine rich repeat (LRR) motifs for pathogen recognition, the cytoplasmic part containing the TLR/IL-1 receptor (TIR) domain for signalling; and the transmembrane domain for fixing the receptor on the cell membrane (Armant and Fenton, 2002).

TLRs are different in their cellular locations. TLRs 1, 2, 4, 5, and 6 are on the cell surface as transmembrane receptors. These cell surface TLRs are responsible for sensing extracellular pathogens. TLR2 can form heterodimers with TLR1 or TLR6 and allow recognising peptidoglycan and lipopeptides from all bacteria and parasites, while TLR4 recognises LPS from gram-negative bacterial

and TLR5 is for bacterial flagellin (Table 1.1). In contrast, TLRs3, 7, 8 and 9 are located intracellularly on the endosomal membrane and recognise intracellular PAMPs of virus and bacterial (Table 1.1). TLR3 is specifically for sensing double-strain RNA viruses, TLR7 and 8 are for single- strain RNA viruses, and TLR9 for pathogen DNA cytosine-phosphate-guanine motifs (CpG) (Kindt et al, 2007). Together, TLR family members recognise a wide-range of PAMPs or DAMPs intra and extracellularly.

Table 1.1 Toll-like receptors classes

Receptor type	Microbial ligands
TLR2/ TLR1 ^a	Triacyllipopeptides
TLR2	Lipoproteins, glycolipids and peptidoglycans of Gram-positive organisms
TLR3	Double-stranded viral RNA
TLR4	LPS ^b of Gram-negative organisms
TLR5	Bacterial flagellin
TLR2/ TLR6	Diacyllipopeptides
TLR7	Guanosine or uridine-rich single-stranded viral RNA
TLR8	Guanosine or uridine-rich single-stranded viral RNA
TLR9	Unmethylated bacterial CpG motifs
TLR10	Possibly similar to TLR1 and TLR6
TLR11	Non-functioning in humans

^a: Toll like receptor, ^b: Lipopolysaccharide.

(Modified from Armant, A., Fenton, J. (2002). Toll-like receptors: a family of pattern-recognition receptors in mammals. *Genome Biology*, 3(8), pp. 3011: 1-6.

1.3.2 TLR signalling pathways

TLRs signal in 5 steps: 1) Microbial PAMPs interact with the LRR motifs of TLRs to form a complex and induce dimerization of TLRs. 2) Intracellular signalling adaptor molecules (MYD88, MAL, TRIF, TIRAP, and/or TRAM) are recruited to TLR via their TIR domains. 3) Several protein kinases. 4) Transcription factors are recruited, and activated via phosphorylation and dephosphorylation by the protein kinases. 5) The activated transcription factors translocated into the

nucleus bind to the target genes and induce the expression of various genes including pro-inflammatory cytokines, chemokines, and surface molecules (Fig 1.4) (Medzhitov,2001).

Based on the usage of adaptor molecule MYD88, TLRs can signal through two different signalling pathways; the MYD88-dependent and MYD88-independent pathways (Akira and Takeda, 2004). MYD88 signalling pathway plays a crucial role in all IL-1R and TLR signal transductions except TLR3 (Medzhitov, 2001). The MYD88-dependent pathway begins with the recruitment of the adaptor protein MYD88 after engagement of TLRs with the specific ligand. MYD88 molecule stimulation is followed by interaction of the TIR domains of both MYD88 and IL-1R-associated kinase (IRAK-4) to form a complexes called the Myddosome (Lin et al, 2010). IRAK-4 phosphorylates IRAK-1, which associates with the RING-domain of E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF-6), activating either TAB or TGF- β activated kinase (TAK-1) complex (Akira et al, 2006). TAK-1/TAB complex activation enhances the I κ B kinase (IKK) complex activity, which induces degradation of I κ B α leading to nuclear translocation of the transcription factor NF- κ B. The translocated NF- κ B induces the transcription of inflammatory cytokines IL-6, IL-8, TNF- α , IL-1 β , and IL-12 and mediators (Wang et al, 2001).

The MYD88-independent pathway is also known as the TIR domain containing adaptor inducing IFN β (TRIF)-dependent pathway (Kawai and Akira, 2006; Jiang and Chen, 2012). Only internalised TLR4 and TLR3 signal through this pathway. Activation of this pathway begins when these TLRs are stimulated by their ligands, leading to recruitment of TRIF molecule, followed by the activation of transcription factor IRF-3. The IRF-3 then translocates into the nucleus and delayed phase NF- κ B activation (Kawai and Akira, 2006). The MYD88-independent pathway mainly leads to the production of type I IFNs that in turn activate STAT1 and induce IFN-inducible genes (Toshchakov et al, 2002). Thus, the MYD88-independent pathway plays an important role in the anti-virus and intracellular infection by inducing type I IFNs.

1.3.3 Function in immune response

TLR signals play a central role in the initiation and activation of innate and adaptive immune responses (Akira et al, 2000). This is because TLRs are widely

expressed by all innate and adaptive immune cells and TLR signals are powerful in immune cell activation.

TLRs orchestrate innate immune defence mechanisms against invading pathogens, in particular the intracellular pathogen elimination in macrophages (Lang, 2005). In macrophages, TLR signals enhance the phagocytosis of pathogens and the formation of phagolysosomes (Doyle et al, 2003). TLR signals also induce the production of antimicrobial mediators including reactive oxygenspecies (ROS) and reactive nitrogen species (RNS); together these will kill the intracellular pathogen. However, microglial activation through TLRs releases chemokines that attract peripheral immune cells into the site of infection in the brain, such as macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1) (Patel et al, 2012). Macrophage activation by TLR4 may also result in proinflammatory cytokines IL-6, IL-12, IL-8, TNF- α , and IL-1 β and chemokine production and plays a pathogenic role in proinflammatory and autoimmune disorders (Chang et al, 2007).

TLR also promote the activation of neutrophils, for instance, enhancing neutrophil phagocytosis, the production of reactive oxygen and nitrogen species as well as the antimicrobial peptides. TLR signals enhance cytotoxicity of NK cells and the production of IFN to kill infected cells and tumour cells (Guo and Zhang, 2012). DCs are the professional APCs in tissue and can initiate T cell activation. TLRs can promote maturation of immature DC to become mature DC, which can provide antigen, costimulatory, and cytokine signals to induce naive T cell priming (Casals et al, 2007).

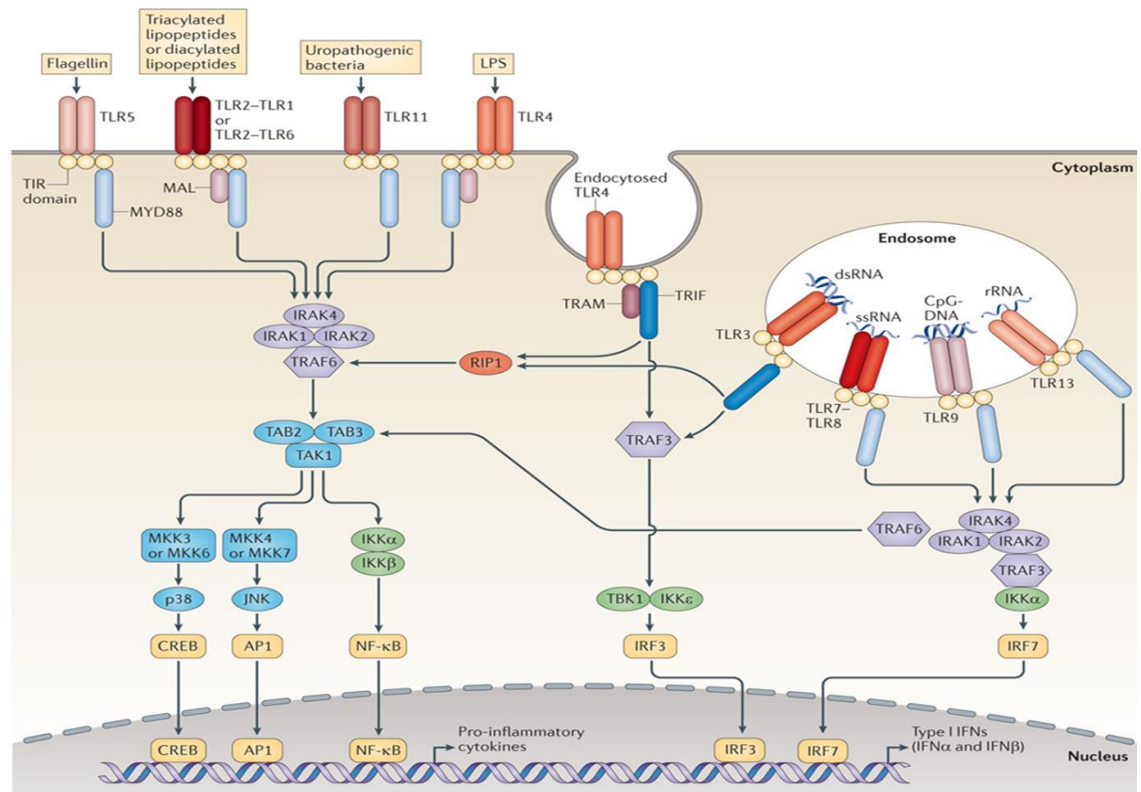


Figure 1.4 Mammalian TLRs signalling

Cell surface TLRs including TLR5, TLR11, TLR4, and the heterodimers of TLR2-TLR1 or TLR2-TLR6 bind to their respective extracellular ligands, whereas TLR3, TLR7-TLR8, TLR9 and TLR13 localized at the endosomes, and these intracellular ones sense microbial nucleic acids. All TLRs signal via the adaptor MyD88, except TLR3 that can function only via TRIF. TLR signalling is initiated by ligand-induced dimerization of receptors, followed by the activation of the TIR domain and adaptor protein MYD88 or TRIF; MyD88 leads to the activation of kinases such as IL-1 receptor-associated kinases (IRAKs). The resulting phosphorylation cascade activates members of TNF receptor associated factor 6 (TRAF6), and ultimately transcription factors NF-κB, JNK and p38 (as well as IRF7 for TLR7 and TLR9). The TRIF pathway stimulates TRAF6, and the transcription factors IRF3, IRF7, NF-κB. Membrane signalling triggers an inflammatory response whereas intracellular TLR signalling leads to antiviral response.

(Adapted from O'Neill, L., Golenbock, D. and Bowie, A. (2013). The history of Toll-like receptors – redefining innate immunity. *Nature Reviews Immunology*, 13(6), pp.453-460).

During the virus infection, the intracellular virus can be recognised by TLR3, 7/8 and 9 in tissue or macrophages. The activation and signalling of intracellular TLRs will lead to the production of type I IFNs to kill the infected virus subsequently (Uematsu and Akira, 2007).

To prevent host tissue destruction from excess production of pro-inflammatory cytokines produced in TLR activated immune cells, type I IFNs are released which in turn upregulate IL-10 production; the autocrine action of IL-10 induces the expression of signal-transducer and activator of transcription (STAT3) which promotes the production of anti-inflammatory mediators (Chang et al, 2007). A range of cytokines, including IL-10, IL-6 and IL-27, are activated by STAT3 signalling (Lang, 2005). Therefore, the release of IL-10 and sustained activation of STAT3 are critical anti-inflammatory feedback mechanisms in macrophages (Kühn et al, 1993; Berg et al, 1995; Takeda et al, 1999; Lang, 2005; Murray, 2005).

1.3.4 TLRs function in adaptive immune cells

Current evidence suggests that both B and T cells express different TLRs. While the function of TLRs on T cells is less understood, their roles in B cell activation and function are well studied. TLR expression in human naive B cells is low to undetectable in general, but the expression can be upregulated with BCR stimulation. In contrast, human memory B cells constantly express TLR9. Mouse B cells express TLR9, and TLR4 is the predominant TLR in naive and memory B cells (Bernasconi et al, 2003; Bourke et al, 2003; Lanzavecchia and Sallusto, 2007).

Mouse B cells stimulated with LPS induces polyclonal expansion, isotype switching and differentiation to plasma cells (PCs) in switched memory B cells in the absence of T-helper cells. However, primary humoral responses can also be induced in response to LPS in naive and un-switched memory B cells (Bourke, 2003; Lanzavecchia and Sallusto, 2007). In humans, CpG stimulation induces naive B cell proliferation, and expression of CD40, CD80, and MHC-II on the cell surface (Jiang and Lederman et al, 2007). In addition, CpG is an excellent agent for inducing cell proliferation in B cells activated by BCR and T- helper cells in vitro, and rescues B cells from abortive proliferation (Krieg, 2000; Ruprecht and

Lanzavecchia, 2006). The CpG alone cannot mediate naive, memory and class switch B cell differentiation into plasma cells, but it can do so in combination with additional signals provided through CD40 or BCR. CpG in combination with IL-4 rescues naive B cells from apoptosis in vitro (Jiang and Lederman et al, 2007).

Research from this lab demonstrated that TLRs can be induced in naive T cells by TCR stimulation and activation (Komai-Koma et al, 2004). TLR signals can replace the costimulatory signals in T cell activation, and enhance T cell proliferation, cytokine production and survival. Intriguingly, TLR signals mainly induce IFN γ and IL-2 production, suggesting that TLRs may predominantly polarise Th1 cells (Komai-Koma et al, 2004).

1.4 Cytokines

Cytokines are small peptides (8-40) kDa, secreted by all nucleated cells, especially immune cells (Thomson and Lotze, 2003). Cells expressing intact cytokine receptors are responsive to cytokine signalling, which is considered the major communication mechanism between cells of the immune system (Marshall, 1992). More than 200 cytokine-like molecules have been identified which work synergistically or antagonistically, that may regulate other cytokine production positively and negatively (Dinarello, 2007). Cytokines are pleotropic in immune response, and they act in autocrine action, paracrine action, and endocrine manner (Tayal and Kalra, 2008).

1.4.1 Cytokine classification

1. Cytokines are classified according to their source, functions and structure. Based on cellular sources, cytokines can be classified into three types:

- Monokines, which are cytokines produced by monocytes and macrophages
- Lymphokines, which are cytokines produced by lymphocytes
- Interleukins, which are cytokines mainly produced by leukocytes

2. According to their function, cytokines can be divided into 4 classes:

- Pro-inflammatory cytokines: produced during inflammation and responsible for inflammatory response
- Anti-inflammatory cytokines: inhibiting inflammation and promoting healing
- Growth factors: necessary for cell growth and survival
- Chemokines: chemoattractant cytokines for attracting cells to the site of inflammation

(Thomson and Lotze, 2003; Dinarello, 2007)

3. Based on structural homology, cytokines can be divided into 9 cytokines families:

- a) The hematopoietic family (IL-2, IL-4, IL-5, IL-13, IL-15, IL-21)
- b) The interferon family (IFN α , IFN β , IFN γ)
- c) The tumour necrosis factor (TNF) family (TNF α , LT α , LT β , RANKL, APRIL)
- d) The IL-1 family (IL-1 α , IL-1 β , IL-1Ra, IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ , IL-36Ra, IL-37, IL-38)
- e) The IL-17 family (IL-17, IL-25)
- f) The IL-10 family (IL-10, IL-22)
- g) The IL-12 family (IL-12, IL-23, IL-35, IL-27)
- h) The TGF family (TGF- α and β)
- i) The chemokine family (CCL1 to 28, CXCL1 to 17, XCL1,2, CX3CL1)

Table 1.2 Functional classification of cytokines and receptors

Functional class	Primary property	Other effects	Examples
Lymphocyte growth factors	clonal expansion	Th1/Th2/Th17 polarization	IL-2, IL-4, IL-7, IL-17, IL-15
Th1 cytokines	↑ Th1 responses	clonal expansion of CTL	IFN- γ , IL-2, IL-12, IL-18
Th2 cytokines Th17 cytokines Pro-inflammatory cytokines	↑ Th2 responses ↑ Th17 responses, IFN γ ↑ inflammatory mediators	↑ antibody production autoimmune responses ↑ innate immune responses	IL-4, IL-5, IL-18, IL-25, IL-33 IL-17, IL-23, IFN- γ IL-1 α , IL-18, TNF- α , IL-12, IL-18, IL-23, MIF, IL-32, IL33, CD40L
Anti-inflammatory cytokines	↓ inflammatory genes	↓ cytokine-mediated lethality, ↓ autoimmune disease	IL-10, IL-13, TGF- β , IL-22, IL-1Ra, IFN α / β
Adipokines	pro-inflammatory	pro-atherogenic, anti-inflammatory	IL-1 α , TNF- α , IL-6, leptin, adiponectin, resistin
gp130 signalling cytokines	growth factors	B cell activation, acute phase	IL-6, CNTF ^c , IL-11, LIF ^c , CT-1 ^c
Nerve growth factors	↑ nerve/Schwann cells	B cell activation	BDNF ^c , NGF ^c
Osteoclast activating cytokines	bone resorption	immune stimulation	RANKL ^c
Colony-stimulating factors	haematopoiesis	pro- and anti-inflammatory	IL-3, IL-7, G-CSF, GM-CSF, M-CSF
Angiogenic cytokines	neovascularization	pro-metastatic	VEGF ^c , IL-1, IL-6, IL-8
Mesenchymal growth factors	fibrosis	pro-metastatic	FGF, HGF, TGF- β , BMP ^c)
Type II IFN	macrophage activation	↑ MHC class II	IFN γ
Type I IFN	anti-viral ↑ MHC class I	anti-inflammatory, anti-angiogenic	IFN α , IFN β
Chemokines ^b)	↑ cellular emigration	↑ cell activation	IL-8, MCP-1, MIP-1 α , others

a) Does not include soluble cytokine receptors such as sTNFRp55, sTNFRp75, sIL-1R type II, IL-18 binding protein, osteoprotegerin. b) The chemokine family includes CC and CXC chemokine with over 30 members. c) BMP, bone morphogenic protein; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; LIF, leukemia inhibitory factor; NGF, nerve growth factor; RANKL, receptor activator of NF- κ B ligand; VEGF, vascular endothelial growth factor.

(Adapted from Dinarello, C. A. (2007). Historical insights into cytokines. *Eur J Immunol*, 37 Suppl 1, S34-45).

1.4.2 The IL-1 family cytokines

Since the cytokines in IL-1 family are closely involved in the study I should like to introduce the family in detail.

The IL-1 family is composed of about 15 members and includes 12 cytokines with agonist activity (IL-1 α and β , IL-18, IL-33, IL-36 α , β , γ , IL-37a, b, c, d, e) and three receptor antagonists (IL-1Ra, IL-36Ra, IL-38) (Vande veerdonk et al, 2015). This family of cytokines is characterized by the β -trefoil domain structure (IL-1like domain) for receptor binding and bioactivity (Dinarello, 2009; Dinarello et al, 2013; Garlanda et al, 2013).

There are several distinct features of the IL-1 family cytokines:

- All the family members have the IL-1-like domain (Garlanda et al, 2013).
- IL-1 family cytokines secreted through unconventional protein secretory pathways, via secretory lysosomes, exosomes or exocytosis vesicles, due to the lack of a signal peptide (Carruth et al, 1991).
- All members signal via a heterodimer receptor complex composed of a receptor and a co-receptor (Lingel et al, 2009).
- IL-1 cytokine functions are self-regulated, either by soluble receptors or antagonising proteins (Garlanda et al, 2013).
- Some of the IL-1 family members function as cytokines when released or as a nuclear factor in the nucleus, for instance IL-33, IL-1 α , and IL-37 (Carriere et al, 2006; Boraschi et al, 2011).

1.4.2.1 IL-1 family cytokines function

The IL-1 family plays a central role in the initiation and development of innate and adaptive immunity and diseases with both proand anti-inflammatory properties. Intriguingly, the function of the IL-1 family is self-regulated by the antagonist, soluble receptor and binding proteins. As illustrated in table 1.2, IL-1 α and IL-1 β are important in the initiation of inflammatory responses and the

polarisation of Th17 cells (Volchenkov et al, 2017). IL-1 receptor antagonist (IL-1Ra) regulates IL-1 function (Arend et al, 1998). IL-18 promotes Th1 responses and Th1 cell development by facilitating IL-12's effect, however, this effect can be inhibited by IL-18 binding protein (IL-18BP). IL-18 is able to stimulate Th2 responses in the absence of IL-12 and IL-15 (Sedimbi et al, 2013 ; Dinarello et al, 2013). IL-33 signals via co-receptor ST2 and IL-1RAcP. It mainly induces type II cytokines, IL-5, and IL-13 in many innate immune cells. IL-33 can also promote Th2 cell function and plays an important role in protection against parasite infection and also allergy and asthma and this induction can be regulated by soluble ST2 (sST2) receptor (Schmitz et al, 2005).

IL-36 is composed of three isoforms, IL-36 α , IL-36 β , and IL-36 γ that share the common receptor complex (IL-36 receptor). IL-36 plays a pathogenic role in lung inflammation and dermatitis (Vigne et al, 2012; Keermann et al, 2015). Both IL-36 receptor antagonist (IL-36Ra) and the IL-36 binding protein IL-38 control IL-36 functions (van de Veerdonk et al, 2015). The latest member of the IL-1 family cytokines is IL-37 with five isoforms IL-37(a-e), it is an important immune-regulator that suppresses inflammatory responses in immunity and in disease (Nold et al, 2010).

Table 1.3 IL-1 Family members

Family name	Name	Receptor/ coreceptor	Property	Synthesized as precursor	Processing required for activity
IL-1F1	IL-1 α	IL-1RI IL-1RAcP	Pro- inflammatory	Yes	No
IL-1F2	IL-1 β	IL-1RI IL-1RAcP	Pro- inflammatory	Yes	Yes
IL-1F3	IL-1Ra	IL-1RI	Antagonist for IL1 α , β	No	No
IL-1F4	IL-18	IL-18R α IL-18R β	Pro- inflammatory	Yes	Yes
IL-1F5	IL-36Ra	IL-1Rrp2	Antagonist for IL36	Yes	Yes
IL-1F6	IL-36 α	IL-1Rrp2 IL-1RAcP	Pro- inflammatory	Yes	Yes
IL-1F7	IL-37	IL-18R α IL-18BP	Anti- inflammatory	Yes	Yes
IL-1F8	IL-36 β	IL-1Rrp2 IL-1RAcP	Pro- inflammatory	Yes	Yes
IL1-F9	IL-36 γ	IL-1Rrp2 IL-1RAcP	Pro- inflammatory	Yes	Yes
IL-1F10	IL-38	IL-1Rrp2	? Antagonist	Yes	? No
IL-1F11	IL-33	ST2 IL-1RAcP	Pro- inflammatory	Yes	No

(Adapted Carta, S., Laveri, R. and Rubartelli, A. (2013). Different Members of the IL-1 Family Come Out in Different Ways: DAMPs vs. Cytokines?. *Frontiers in Immunology*, 4.)

1.4.3 IL-37

IL-37 was discovered in silico research in 2000 (Moretti et al, 2014). IL-37 gene was also called IL-1H4, IL-1RP, or IL-1F7.

1.4.3.1 IL-37 isoforms

The human IL-37 gene is located on chromosome 2 between the IL-1B and IL1F9 genes (Fig 1.5). IL-37 gene in mice is missing, thus mice do not express IL-37 (Boraschi et al, 2011). The IL-37 gene consists of 6 exons and can encode five isoforms (a, b, c, d, and e) by mRNA alternative splicing. Exons 1-3 encode the N-terminal domain of IL-37 isoforms. Exons 4-6 encode the C-terminal domain of IL-37 isoforms containing 12 putative β -strands to form the β -trefoil protein structure, which is the characteristic of the IL-1 family (IL-1 like domain) and can bind to IL-1Rs for signalling (Fig 1.8)

IL-37b is the longest among the IL-37 isoforms encoded by exons 1, 2, 4, 5, and 6. A caspase-1 cleavage site is detected in its N-terminal domain between the amino acids asparagine (D²⁰) and glutamine (E²¹), and this cleavage site exists in all IL-37 (b-e) isoforms except IL-37a. This may give a mature IL-37b starting at E²¹. In contrast, IL-37a is encoded by exons 3, 4, 5, 6; and it is the only isoform using exon 3 to encode protein (Fig 1.6) (Taylor et al, 2002). IL-37a lacks the caspase-1 cleavage site. Instead, it has a putative elastase cleavage site at the N-terminus upstream of the β -trefoil structure between leucine (L²¹) and arginine (R²²) (Fig 1.7) (Dinarello et al, 2016). In addition, IL-37a has a putative NLS for protein translocation into the nucleus, suggesting that IL-37a may be a nuclear protein (Taylor et al, 2002; Boraschi et al, 2011). IL-37d is encoded by exons 1 and 4 to 6. IL-37c is encoded by exons 1, 2, 5 and 6 and IL-37e is encoded by exons 1, 5 and 6 (Boraschi et al, 2011). The same as IL-37b, all the 3 isoforms can be processed by caspase-1.

Based on whether or not carrying the IL-1-like domain, the isoforms can be divided into two functional groups; the isoforms IL-37a, b, and d (i.e. those encoded by the complete exon 4-6) will therefore be expected to bind to the same receptor and trigger the same range of biological effects. In contrast, IL-37c and e lack the key exon 4 and are expected to give rise to a partial IL-1-like

B-trefoil structure and their functions are unknown (Taylor et al, 2002; Boraschi et al, 2011). This protein/structural evidence suggests that the isoforms may vary in function.

Another important domain in the IL-37 gene is the instability element within exon 5 represented by a conserved ten-nucleotide homology box that is found in the open reading frame of IL-37 gene (Bufler et al, 2004; Quirk and Agrawal, 2014). The sequence can control IL-37 RNA stability and can be inactivated by inflammatory signals. In resting cells, IL-37 mRNA will be rapidly degraded, and can be induced by inflammatory signals, for instance LPS stimulation. Thus, this instability element limits IL-37 mRNA and protein levels and ensure that it is induced only during inflammatory but not in resting conditions (Bufler et al, 2004).

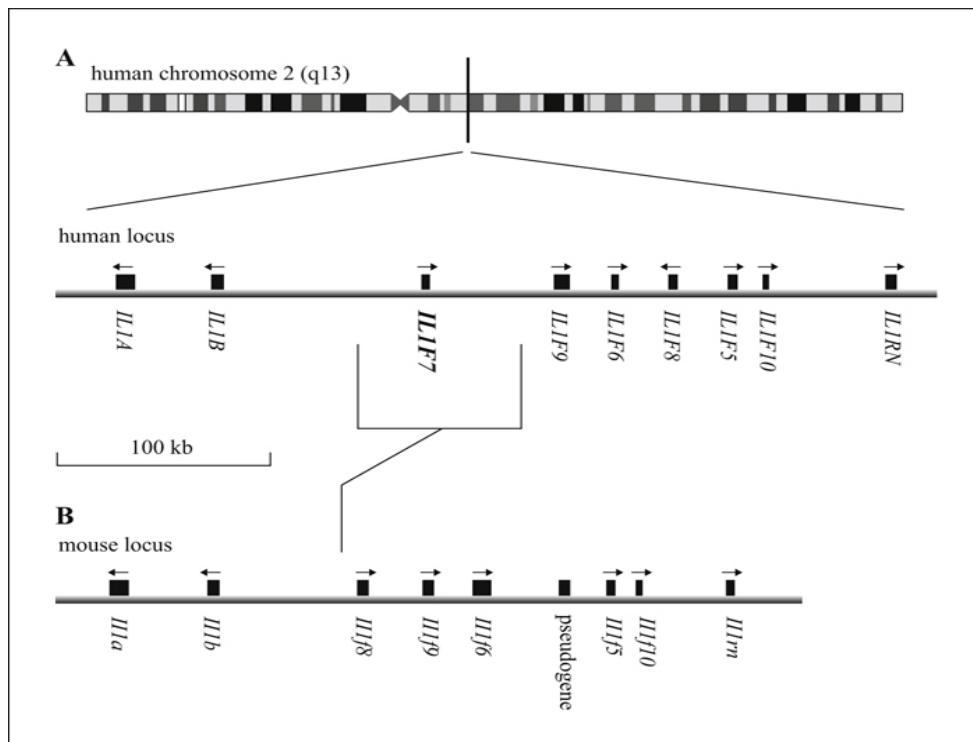


Figure 1.5 Genomic location of IL1F7 gene in human and mouse.

Panel A shows the genes of IL-1 family locus on human chromosome. 2) Panel B illustrates the genes of IL-1 family locus on mouse chromosome 2. The IL1F7 (IL-37) gene is between IL1B and IL1F9 in human. In mouse it is absent, instead IL-1F8 is localised at the expected IL-1f7 position.

(Adapted from Boraschi, D., Lucchesi, D., Hainzl, S., Leitner, M., Maier, E., Mangelberger, D., Oostingh, G., Pfaller, T., Pixner, C., Posselt, G., Italiani, P., Nold, M., Nold-Petry, C., Bufler, P., Dinarello, C. (2011). IL-37: a new anti-inflammatory cytokine of the IL-1 family. *Eur Cytokine Netw*, 22(3), pp. 127-47).

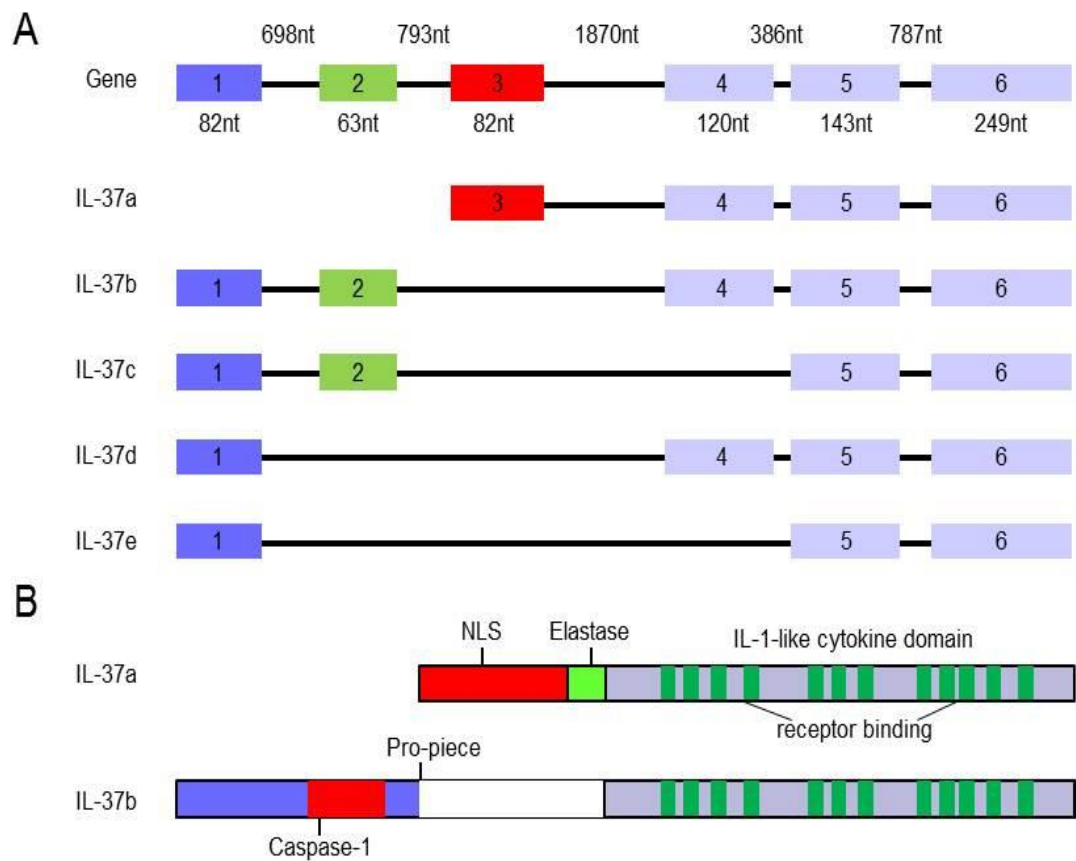


Figure 1.6 Structure of the IL-37 gene

IL-37 gene is composed of 6 exons (1-6) as shown. A) Illustrates IL-37 gene and related 5 isoforms; B) illustrates the protein domains of IL-37a and IL-37b isoforms, IL-37a possesses NLS and elastase putative site in exon 3, while exons 4-6 encode the receptor binding sequence. IL-37b has exon 1, which contains a caspase-1 cleavage site, while IL-37b shares exons 4-6 with IL-37a and express the receptor-binding site. Size of the exons indicated as numbers down each exon. More info

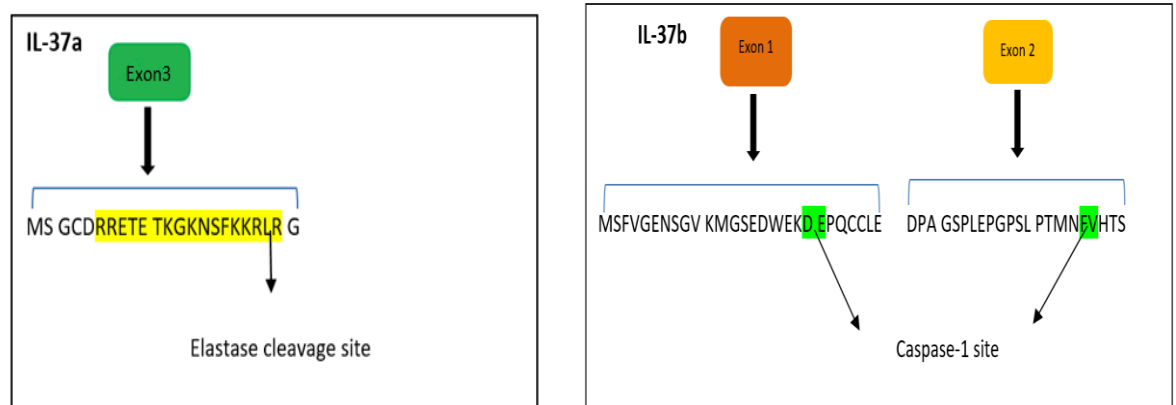


Figure 1.7 Cleavage sites in predicted amino acids sequences of exon 1, 2, and 3

The figure illustrates the enzyme cleavage sites of IL- 37a and b. A) Exon 3 expressed exclusively by IL-37a isoform contains a unique elastase cleavage site encoded by exon 3, between leucine (L²¹) and arginine (R²²) (the black arrow). The location of NLS highlighted by yellow colour. (B) Illustrates the two caspase-1 cleavage sites of IL-37b, encoded by exon1 and exon 2, respectively (indicated by arrows). The first is between asparagine (D²⁰) and glutamine (E²¹) and the second is between phenyl- alanine (F⁴⁵) and valine (V⁴⁶).

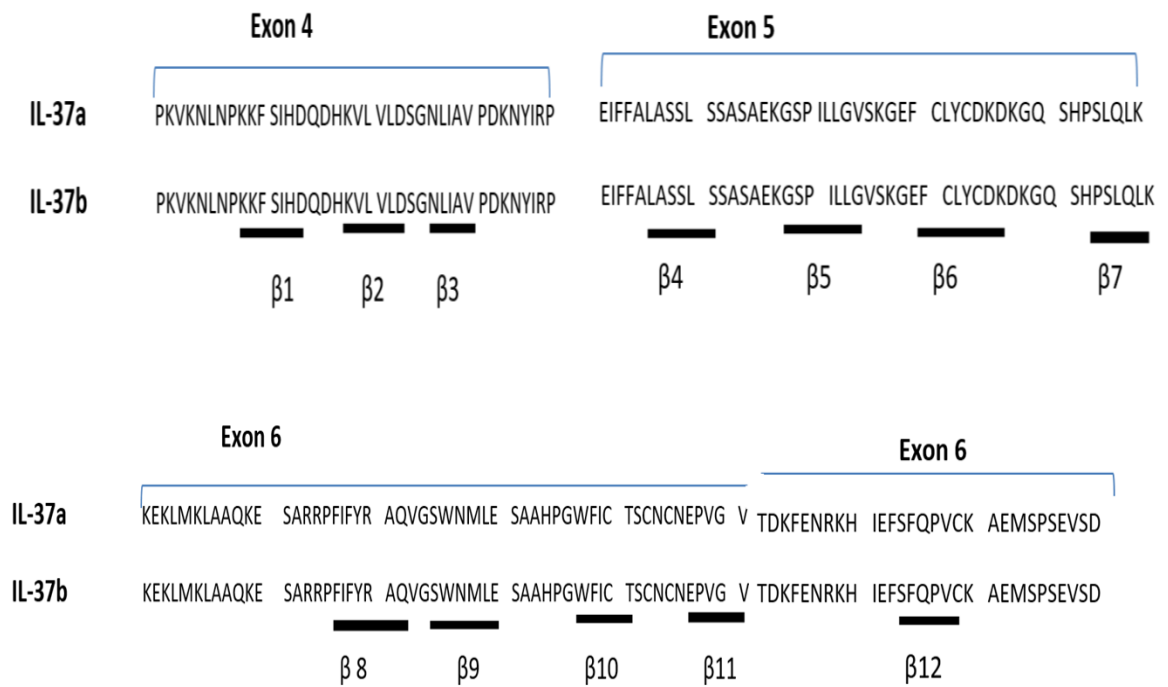


Figure 1.8 The predicted amino acids encoded by exons 4, 5, and 6

Both IL37a and IL37b isoforms share exons 4-6 that encode the trefoil domain. The lines underneath each group of amino acids represent β strand. There are 12 β strands. Together these form the β trefoil structure of IL-37

1.4.3.2 IL-37 Maturation

IL-37 is translated as a full length immature or pro-IL-37 protein. The immature IL-37 isoforms except IL-37a and contain a caspase-1 cleavage site and can be processed efficiently by caspase-1 into mature IL-37. Mature IL-37b beginning with (EPQC) amino acids sequence at the N-terminal suggests that the predicted cleavage site of caspase-1 occurs between aspartic acid (D²⁰), and glutamic acid (E²¹) (Fig 1.8) (Kumar et al, 2002). Although IL-37 may also contain a second caspase-1 cleavage site encoded by exon 2, the main caspase-1 cleavage site may be located around D²⁰, because mutation of aspartic acid at position (D²⁰) markedly reduced caspase-1-mediated maturation processing of pro-IL-37 (Bulau et al, 2014). However, the caspase-1-mediated processing in other IL-37 isoforms carrying the caspase-1 cleavage site is largely unknown, but is expected to have the same effect because of their similarity in protein sequences. IL-37a is a unique isoform that carries a putative elastase but not caspase-1 cleavage site at its N-terminal. This suggests that IL-37a differs from other isoforms in maturation. This is under investigation in our lab. Interestingly, different from IL-1 β and IL-18, maturation seems not important in the function of IL-37, because both mature and immature IL-37 can bind to and signal via the IL-37 receptor complex composed of IL-18R α and IL-1R8 (Nold et al, 2010). The physiological and pathological impact of maturation on IL-37 function is still unclear.

1.4.3.3 Secretion and nuclear location

The same as other members in the IL-1 family, IL-37 isoforms do not have a signal sequence that facilitates the protein secretion out of the cells. Both pro and mature IL-37 can be secreted outside the cell by unknown mechanism, independent of the conventional endoplasmic reticulum and Golgi apparatus (Dinarello et al, 2016). This suggests that different from IL-1 and IL-18, maturation is not required for their secretion (Nold-Petry et al, 2015).

It has been demonstrated that both IL-37b and IL-37d can also translocate into the nucleus (Luo et al, 2017; Zhao et al, 2018), While the detailed mechanism is still unknown. It has been shown that only the mature but not the immature form of IL-37b can do so by binding to and co-translocating into nucleus with

Smad3 (Nold et al, 2010; Dinarello et al, 2013). It has been evidenced that caspase-1 is necessary for IL-37b maturation and translocation to the nucleus, as a caspase-1 inhibitor abolished completely IL-37 maturation and nuclear translocation (Fig 1.9) (Kumar et al, 2002).

Different from other IL-37 isoforms, the putative nuclear localizing sequence (NLS) sequence in pro-IL-37a predicts that immature IL-37a may be able to translocate to the nucleus (Nold et al, 2010). The research result from this lab confirmed that the NLS has a function, which enables immature IL-37a to translocate to the nucleus without maturation (Dr. Xu unpublished result). Thus, IL-37 isoforms may be also dual functional, able to serve as both a nuclear factor and a cytokine by different mechanisms.

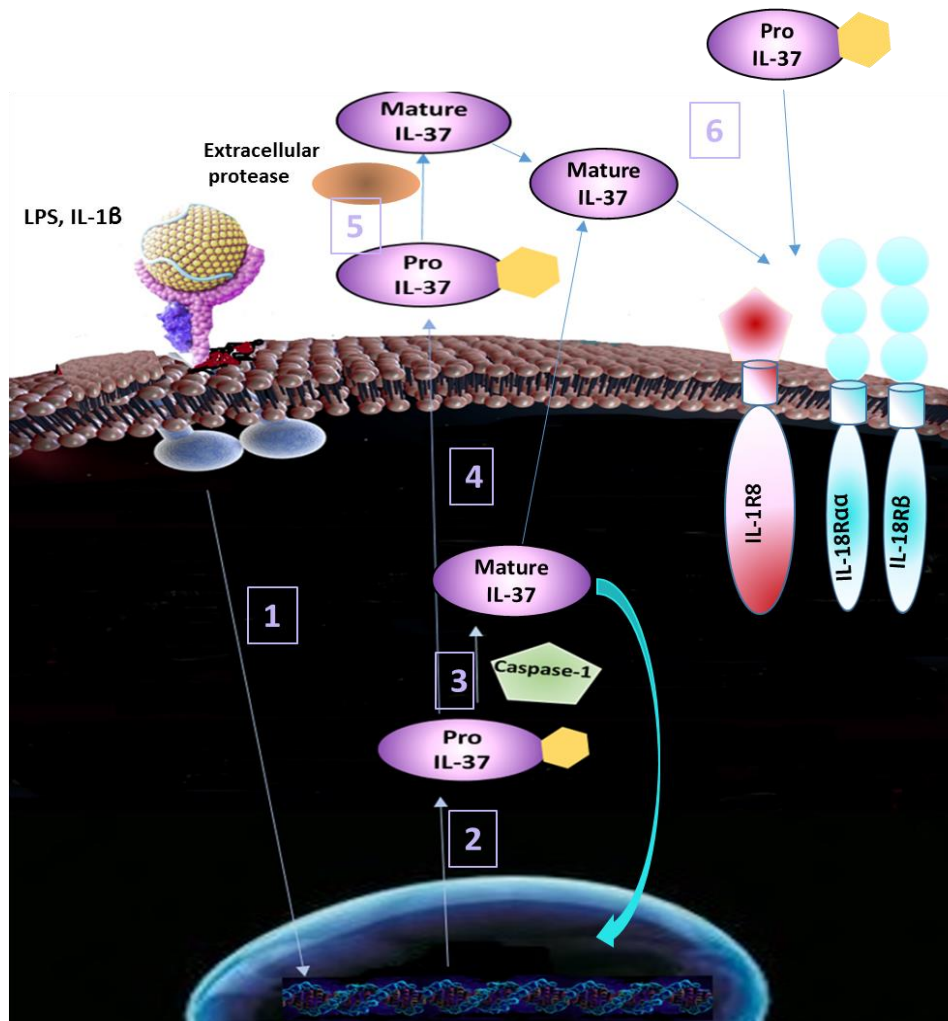


Figure 1.9 IL-37 maturation

The figure illustrates the induction and maturation of pro-IL-37. (1) IL-1 β and LPS activate immune cells which express IL-37. (2) Upon stimulation, IL-37 expression increases and is released into the cytoplasm as pro-IL-37. (3) Caspase-1 enzyme catalyses pro-IL-37 into mature IL-37. (4) Mature and pro-IL-37 are able to release into the extracellular space. (5) pro-IL-37 can be catalysed by extracellular protease into mature IL-37. (6) Mature and pro-IL-37 can both bind to the IL-37 receptor complex and trigger signalling.

(Modified from Dinarello, A., Nold-Petry, C., Nold, M., Fujita, M., Li, S., Kim, S., Bufler, P. (2016). Suppression of innate inflammation and immunity by interleukin-37. *Eur J Immunol*, 46(5), pp.1067-81)

1.4.3.4 Dimerization

The study of crystal structure of IL-37b indicates that IL-37 can form a head-to-head homodimer, suggesting a configuration that is unique in IL-1 superfamily cytokines. Both pro (IL-37⁽¹⁻²¹⁸⁾) and mature (IL-37⁽²¹⁻²¹⁸⁾ or IL-37⁽⁴⁶⁻²¹⁸⁾) protein can form homodimers. The key amino acids that are involved in the dimer formation have also new identified. IL-37b monomers form dimers through the formation of hydrogen bonds between Tyr⁸⁵ and Arg⁸⁷, and this interaction is protected by an ionic gate provided by another two amino acids Lys⁸³ and Asp⁷³ (Ellisdon et al, 2017). Moreover, Tyr⁸⁵, amino acid is highly conserved, suggesting a key role in stabilizing the dimer. Isoforms IL-37c and IL-37e lack the dimer interface amino acids. Due to the absence of strands B1 to B3, suggesting that these isoforms do not dimerize (Ellisdon et al, 2017).

Furthermore, the homodimer formation mainly occurs when the protein concentration is higher than 1.35 μ M (Ellisdon et al, 2017). Importantly, the homodimer formation reduced or even abolished IL-37-mediated suppressive function in vitro and in vivo (Kumar et al, 2002; Ellisdon et al, 2017). Whereas the detailed mechanism is still not fully understood, this may represent a novel way for the self-regulation of IL-37 function. Together with the RNA instability element in IL-37 gene, these suggest that IL-37 expression and function are highly self-regulated.

1.4.3.5 Regulatory effect of IL-37 on immune cell function

Growing evidence has demonstrated that IL-37 is an immune regulatory cytokine and suppresses innate and adaptive immune cell function via receptor-dependent and independent pathways (Nold-Petry et al, 2015). This is attributed to its dual functions, as a nuclear factor and a cytokine.

1.4.3.5.1 IL-37 receptor-dependent pathway

As a cytokine, IL-37 signals via the receptor complex consisting of IL-18R and IL-1R8. IL-37 binds to IL-18R α first at the extracellular space, and then recruits IL-1R8 receptor to form a ligand/receptor complex for intracellular signal transduction (Nold-Petry et al, 2015).

The interaction between IL-37 and IL-18R α is due to IL-37 sharing similarity in amino acids with the IL-18, in particular the two amino acids Glu and Lys that are conserved in IL-18 and IL-37 for the IL-18R α binding (Bufler et al, 2002; Sharma et al, 2008; Bulau et al, 2014). However, the initial interaction between IL-37/IL-18R α is not sufficient to trigger the intracellular signal transduction, therefore the IL-1R8 is recruited to the IL-37-IL-18R α complex to form the tripartite complex on the cell surface and to elicit downstream signal transduction (Fig 1.10) (Bufler et al, 2002; Garlanda et al, 2009; Quirk et al, 2014; Nold-Petry et al, 2015). IL-1R8 is an orphan receptor of IL-1R family (Garlanda et al, 2009). Many reports demonstrated even before the finding of IL-37 that IL-1R8 (sigirr) is a negative immune regulator in immunity and disease (Li et al, 2015). While the detailed mechanism by which IL-1R8 regulates immune response is still lacking, it may do so via its mutation in the TIR domain (Riva et al, 2012). TIR domain, in particularly the conserved amino acids (Ser⁴⁴⁷ and Tyr⁵³⁶), are essential for signal transduction in all the IL-1R family (Garlanda et al, 2013). It has been revealed that IL-1R8 carries mutations in the TIR domain in which the conserved amino acids Ser⁴⁴⁷ and Tyr⁵³⁶ are replaced by the Cys²²² and Leu³⁰⁵ (Riva et al, 2012). These mutations in IL-1R8 tail can still recruit adaptor MYD88 but it prevent the recruitment and activation of downstream protein kinases (Polentarutti et al, 2003; Lech et al, 2007).

Thus, the IL-18R α is important for the initiation of IL-37/receptor complex formation and IL-1R8 for triggering IL-37 signalling. The importance for both the receptors in IL-37 function and signalling has been evaluated by using neutralising antibody siRNA knock down and gene deficient mice of IL-18R α or IL-1R8 and showed that the anti-inflammatory signal transmission of IL-37 requires both receptors (Nold-Petry et al, 2015).

Because of the regulatory effect of IL-1R8 on MYD88 function, it was thought originally that IL-37 may only regulate IL-1 and TLR signalling (the first IL37 paper). Indeed, it has been shown that IL-37b is critical in the inhibition of IL-1 and TLR by affecting the MYD88-dependent pathway and the production of inflammatory cytokine/mediators, including TNF- α , IL-1 and IL-6 (Ye et al, 2014). This plays a central role against extracellular infections and

inflammation. However, its role in the regulation of the TRIF-dependent pathway is less studied.

Interestingly, growing evidence suggests that the IL-37-mediated receptor-dependent immunosuppressive capability may extend beyond the IL-1/TLR signalling and can also suppress inflammatory response induced by many other cytokines, for example TNF α , IL-6 and IL-17 (Nold-Petry et al, 2015). IL-37 may do so by inhibiting many protein kinases, including Fyn, TAK1, MAPK, and the transcription factor NF- κ B, STAT3 and cell metabolic molecule mTOR, depending on the stimuli (Petry and Rudloff et al, 2015). Thus, via the receptor-dependent pathway, IL-37 has a wide range of effects in the regulation of major inflammatory pathways in immunity.

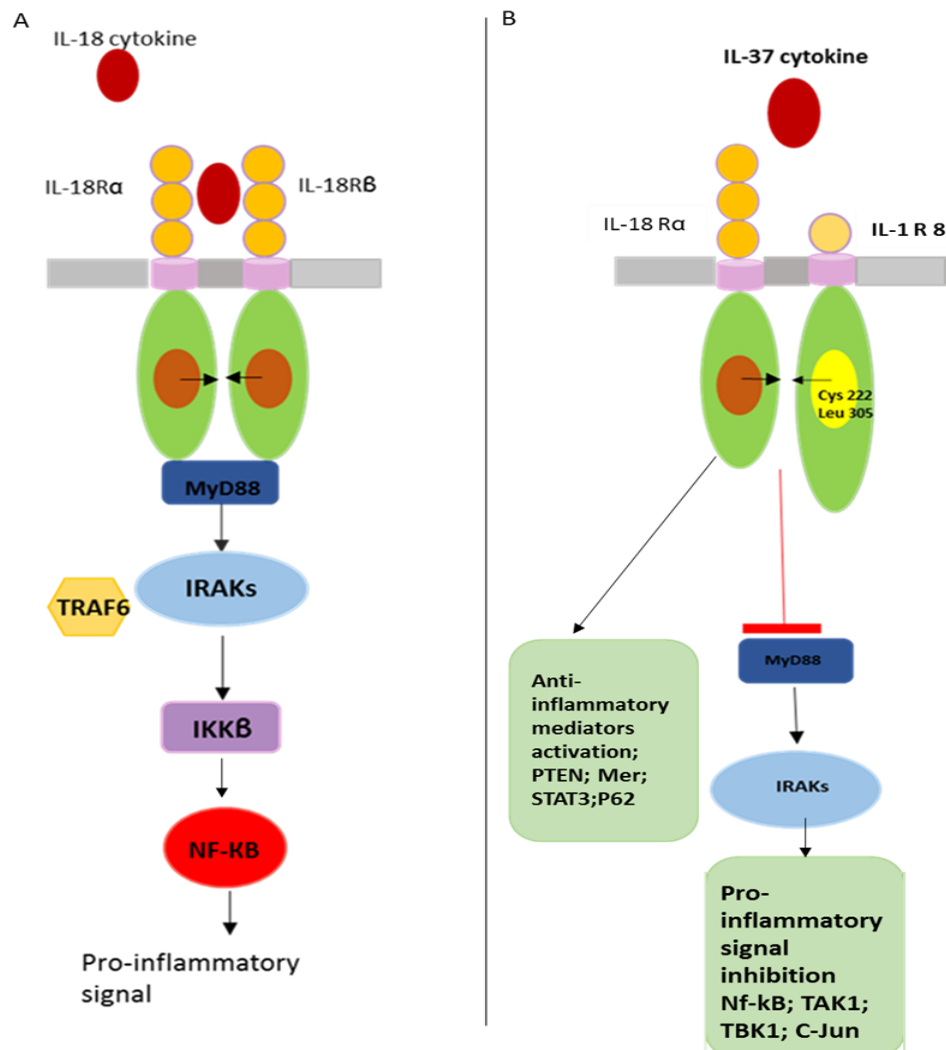


Figure 1.10 IL-37 receptor-dependent signalling pathway

The figure illustrates IL-37 receptor dependent signal. A) The classical pathway of IL-18 receptor. IL-18 binding to IL-18 α , followed by IL-18 β chain activation and signal transduction results in pro-inflammatory mediator release. B) IL-37 receptor dependent-pathway. Extracellular IL-37 binds to IL-18 α first, followed by the tripartite complex formation (IL-37-IL-18 α -IL-1R8) and triggers anti-inflammatory signalling that inhibits downstream pro-inflammatory response.

1.4.3.5.2 IL-37 receptor-independent signalling.

Another important feature of IL-37 isoforms is their receptor-independent regulatory capability in inflammatory gene expression. It has been shown that both IL-37b and IL-37d are able to suppress inflammatory cytokine production in cells without the IL-37 receptor (Luo et al, 2017; Zhao et al, 2018). While the precise mechanism is still unclear, in particular for IL-37d, this is mainly due to their capacity for nuclear translocation by different mechanisms. It has been reported that IL-37b nuclear translocation requires active caspase-1, Smad3, and mature IL-37b but not pro-IL-37b is able to translocate into cell nucleus (Fig 1.11) (Zhao et al, 2018). This was evidenced by: 1) Caspase-1 inhibition prevents IL-37 maturation, nuclear translocation and immune suppressre function. 2) Mutation in the caspase-1 cleavage site (D²⁰) prevents IL-37b translocation to the nucleus (Bulau et al, 2014).3) Smad3-inhibitor prevents IL-37 nucleas translocation and ability to suppress inflammation in vitro and in vivo (Grimsby et al, 2004; Bulau et al, 2014; Dinarello et al, 2016).

Thus, it is possible that stimulation with IL-1 or TLR signals increases IL-37 precursor synthesis in the cytoplasm. In addition, LPS or IL-1 also induces caspase-1 expression. Caspase-1 then catalyses pro-IL-37 into mature IL-37 which binds to phosphorylated-Smad3 to form a IL-37- Smad3 complex which translocates to the nucleus. However, how the Smad3-IL-37 complex regulates gene expression in the nucleus is largely unknown. As Smad3 is also required for TGF- β -mediated immunosuppression, it is possible that IL-37 may also employ the TGF- β Smad3 signalling pathway to control downstream gene expression (Grimsby et al, 2004).

In contrast, we found that pro-IL-37a is able to translocate into cell nucleus via its NLS (unpublished result). However, the effect of nuclear pro-IL-37a on inflammatory gene expression is largely unknown and currently under investigation.

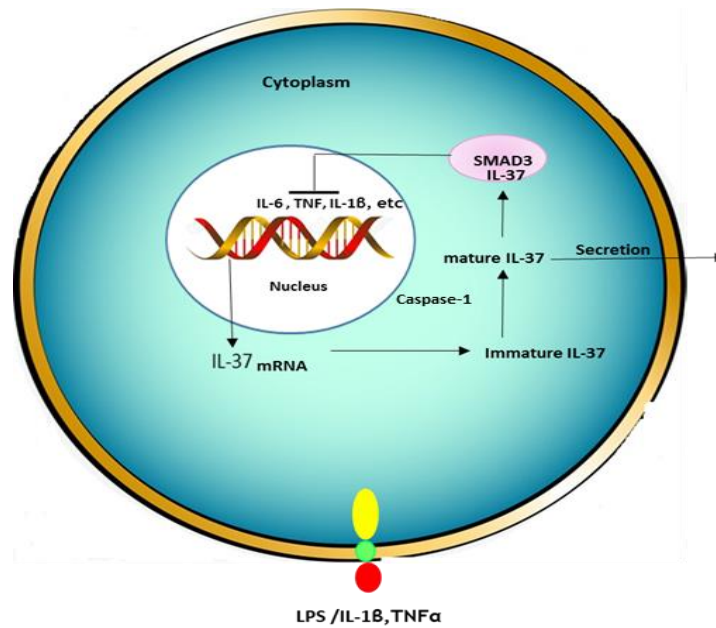


Figure 1.11 IL-37 receptor independent signalling pathway.

Pro IL-37b expression is induced in the response to pro-inflammatory stimuli (TLR agonists, IL-18; TNF). The precursor is processed by caspase-1; both the precursor and the mature forms of IL-37b can be released from the cells via non-classic secretory pathways. The mature IL-37b binds to phosphorylated Smad3 and translocates to the nucleus, where it inhibits transcription of Pro-inflammatory mediators.

1.4.3.6 IL-37 expression and function in human diseases

The isoforms of IL-37 are widely expressed in different immune cells and different tissues (Wang et al, 2018). The expressions of IL-37 isoforms are induced by inflammatory signals, including TLR and inflammatory cytokines (Nold-Petry et al, 2015). IL-37a, IL-37b, and IL-37c expressed in stimulated B cells monocytes, skin keratinocytes, epithelial cells, natural killer (NK) cells. Interestingly, IL-37 isoform expression differs in different tissues; IL-37a is the only isoform that expressed in the brain, while only IL-37b expresses in kidney, IL-37c specifically in the heart; and IL-37d only expressed in the bone marrow and testis (Wang et al, 2018).

IL-37 expression is low in normal cells and tissues, but the expression levels can be changed in disease conditions. More importantly, the cytokine exerts regulatory effect in many diseases including endotoxemia, autoimmune, inflammatory diseases and cancers (Wang et al, 2018). The function of IL-37 in immunity and disease will be discussed in more details in Chapter 6.

1.4.3.7 The effect of IL-37 on LPS-induced endotoxemia.

Endotoxemia is a critical condition caused by LPS from gram-negative bacteria infection. Severe endotoxemia may lead to endotoxic shock and death due to multiple organ failure (Li et al, 2017). This is due to the over-activation of TLR4 by high doses of LPS inducing inflammatory mediator production, including IL-6, IL-8, IL-1, TNF- α , histamine, and oxygen free radicals which in turn lead to septic shock (Wang et al, 2018). Endotoxemia is the best studied IL-37-regulated disease (Li et al, 2015). Current evidence shows that both IL-37b and IL-37d are able to control LPS-induced endotoxemia in mice (Li et al, 2017).

IL-37b transgenic mice had reduced sub-lethal dose of LPS-induced tissue inflammation and hypothermia compared to the wild-type mice (Bulau et al, 2014). Pro-inflammatory cytokine production is also reduced. Thus, IL-37b is a potent repressor of inflammation and protects against endotoxemia through inhibiting the expression of pro-inflammatory cytokines (Li et al, 2015; 2017).

Recent evidence suggests that IL-37d can also protect against endotoxemia in mice (Zhao et al, 2018). In vitro, bone marrow-derived macrophages from IL-37d transgenic mice express less pro-inflammatory cytokines, IL-1, IL-6 and TNF- α , after LPS stimulation, compared to wild type (wt) cells. In vivo, compared to wt mice, IL-37d transgene protects the mice against a lethal dose of LPS challenge (30 mg/kg), reduced mortality and hypothermia (Nold-Petry et al, 2015). The expressions levels of IL-6, TNF- α , IL-1 β , IFN- γ , IL-17a, and chemokine MCP-1 in IL-37d-transgenic (IL-37d-tg) mice were markedly reduced compared with wt mice. However, the underlying mechanism by which IL-37 isoforms suppress LPS shock may be different.

It has been shown that IL-37b can regulate the LPS response via IL-37 receptor-dependent and independent pathways in vitro using cells expressing or not IL-37b and receptor IL-1R8 in a RNA-sequencing assay (Nold-Petry et al, 2015). IL-37 signalling in IL-1R8-dependent pathway activates anti-inflammatory and metabolic mediators including Mer, PTEN, PG2 (dok), GSK-3 β , STAT3, FOXO-1, HD1-5. In addition, the IL-1R8 dependent pathway also inhibits the metabolic check-point mTOR and other inflammatory mediators such as Fyn, PBP, TAK1, TBK1, c-jun, I κ B proteins. NF- κ B pathway is also downregulated by IL-37 using receptor dependent pathway (Nold-Petry et al, 2015).

IL-37b can also regulate important LPS-induced kinases and transcription factors activity independently from the receptors in vitro using RNA-sequencing assay (Nold-Petry et al, 2015). In this pathway, IL-37 significantly down regulates the expression of many inflammatory genes CADTK, Raf-1, KSR, MKK1, MKK6, MKK4, MKK7, ERK1, ERK3, ATF-2, SYK, FADK, STAT5 α , STAT5 β , SHP-2, PAK1, Vav1, S6k-B, IR and IGF-1 receptor. This suggests that IL-37b can regulate inflammatory pathways without the receptor. While this may contribute to IL-37b-mediated regulatory effect, it is unknown how IL-37 does that. Although IL-37 can regulate LPS responses independently from IL-1R8, it has been demonstrated that the receptor-dependent pathway is required for the IL-37-mediated anti-inflammatory effect in endotoxemia since transgenic IL-37 transgenic mice without IL-1R8 completely failed to protect against LPS challenge (Nold-Petry et al, 2015). This result is important to show the relative importance of the two pathways in IL-37-mediated effects. How IL-37d regulates LPS response is less

clear, it has been suggested that IL-37d mediated protective effect is largely receptor-independent in vitro. IL-37d may do this by interacting with Smad3 and promoting nuclear translocation of Smad3 (Zhao et al, 2018). However, whether this is also the case in vivo is unknown. IL-37b is also able to regulate inflammatory bowel disease (IBD). The expression level of IL-37b is markedly high in the epithelial cells of the inflamed mucosa of both Crohn's disease (CD), and ulcerative colitis (UC) patients, while it is not detected in normal colonic mucosa (Imaeda et al, 2013). Using an experimental colitis model, IL-37-tg mice exhibit improved tissue inflammation and clinical scores compared to wt mice (Bello et al, 2018). The reduced inflammatory response may be due to IL-37 targeting the key inflammatory NF- κ B pathway in the inflamed bowel mucosa. IL-37 is also highly expressed in patients with rheumatoid arthritis (RA) compared with healthy controls. Local intra-articular injection of rIL-37 results in delayed disease onset and ameliorates the clinical symptoms of arthritis, mainly through the inhibition of Th17 cell proliferation and the production of inflammatory cytokines, particularly IL-1 β and IL-6 (Ye et al, 2014). IL-37 also suppresses allergic inflammation by abrogating the Th2 response in asthma. Local administration of IL-37 significantly reduces the inflammatory cell infiltration in lung tissue in a murine model of asthma as well as decreasing Th2 cytokine production in lung (Zhang et al, 2017). Intriguingly, IL-37 plays a prominent therapeutic role in several types of cancers. IL-37 suppresses cervical cancer (CC) cell proliferation and invasion in vitro. In CC IL-37, down regulates of key tumorigenic factor STAT3 expression and phosphorylation. Consequently, IL-37 may be a new anticancer cytokine that offers a potential treatment for CC through down regulation of STAT3 expression and phosphorylation (Wang et al, 2015). Colon cancer tissues express IL-37 barely, but IL-37 overexpression in colon cancerous tissues suppresses cancer cell proliferation, migration, invasion, and colony formation by suppressing the expression of β -catenin. In mice colon cancer model, transgenic expression of IL-37 increases cancer cell sensitivity to chemotherapy drugs, suggesting that IL-37 might be a novel therapeutic regulator in colon cancer (Yan et al, 2017). IL-37 also reduces myocardial injury in ischemia reperfusion through suppressing the pro-inflammatory cytokines and chemokines production and reducing infiltration of neutrophils (Wu et al, 2014). All these result in decreased cardiomyocyte apoptosis and ROS generation.

1.5 Recombinant protein production and application

Recombinant proteins have a wide range of applications in clinical treatment and in research. Successful recombinant protein production depends on several factors (Rosano and Ceccarelli, 2014).

1.5.1 Recombinant protein expression systems

1.5.1.1 Host cells

The cellular host for recombinant protein production can be mammalian, insect cells or microorganism systems including bacteria, yeast, filamentous fungi and unicellular algae. The host selection depends on the nature and quality of the recombinant protein. For instance, eukaryotic cells can be used for protein expression if post-translational modifications (such as protein glycosylation) are necessary. Otherwise, a prokaryotic system is preferred (Demain and Vaishnav, 2009; Rosano and Ceccarelli, 2014). Other factors including post-translational modifications of the functional recombinant product, cost, protein yield, toxicity of the protein and secretion of the protein are all critical for the host cell selection since each host system has advantages and disadvantages (Demain and Vaishnav, 2009; Adrio and Demain, 2010).

1.5.1.2 Expression vector

Recombinant protein gene can be expressed from a plasmid vector, which expresses the protein in the host cells. An expression vector contains the interested protein gene (cDNA) which is substituted by the upstream promotor, ribosome-binding site, and downstream stop sequences in the expression vector. Vector specificity is governed by the host system and choosing an appropriate promotor controls the expression of the protein, for instance inducible promoters are needed to avoid the toxicity of recombinant protein to the host; in contrast, constitutive promoters do not need inducer agent (Overton, 2014). The addition of tags to the expressed protein gene is important for recombinant protein detection and purification; and most of these tags not only do not affect the protein folding or secretion but also improve the expressed protein solubility and stability. In addition, tags provide strong affinity for the chromatography medium such as polyhistidine tags that consists of four to 10 histidine residues;

other tags include glutathione S-transferase (GST), maltose-binding protein (MBP) and streptavidin tag strep-tag II (Oliveira and Domingues, 2017).

1.5.1.3 Recombinant protein purification

The purification of tagged recombinant proteins is carried out through immobilised metal affinity chromatography (IMAC) technique with almost a successful single step and different metal affinity chromatography used for different tagged proteins. IMAC can be used as manual or automated system for small and large-scale purification of tagged proteins respectively. Since manual purification is time-consuming, the automated purification system, such as the ÄKTA chromatography systems, is used for large-scale protein preparations with reproducible results. IMAC is based on the interaction between specific amino acid side chains in the tagged recombinant protein and a transition metal ion immobilized on a matrix. This transitional ion could be di-valent ions including Co^{+2} , Ni^{+2} , Cu^{+2} , or Zn^{+2} or trivalent cations such as Al^{+3} , Ga^{+3} , and Fe^{+3} , or tetravalent such as Zr^{+4} depending on the application. Di-valent ions are used for His-tagged proteins, while tri and tetravalent ions are used for phosphoproteins and phosphor peptides (Waugh, 2005; Carson et al, 2007). Soluble recombinant proteins produced in E-coli can be purified by IMAC directly. However, the insoluble protein products in the inclusion bodies can also be efficiently purified as denatured soluble protein using denaturing purification conditions such as high concentrations of urea or guanidinium hydrochloride. The purified denatured proteins can then be renatured by replacing the denaturing reagents with balanced buffers via dialysis (Structural Genomics Consortium, 2008).

1.5.2 The applications of recombinant protein

Recombinant proteins are widely used clinically and in research in different fields.

1.5.2.1 Medicine

Several recombinant proteins are used clinically as effective therapeutics for several fatal metabolic disorders or endocrine dysfunctions, such as insulin for the treatment of type I diabetes and clotting factor VIII for haemophilia, respectively (Hermeling et al, 2004; Akash et al., 2013a, 2013d). Bioactive

recombinant proteins may serve as therapeutic drugs to induce beneficial immune responses; for instance recombinant IFN α for hepatitis C treatment, and recombinant erythropoietin to treat chronic anaemia (Corwin et al, 2002; van Zonneveld et al, 2004; Akash et al, 2015). Recombinant therapeutic proteins with specific targeting activity such as Ibritumomab tiuxetan (anti-CD20 recombinant antibody) and anakinra (IL-1 antagonist) are used to treat transformed non-Hodgkin's lymphoma and rheumatoid arthritis respectively through different mechanisms, including interfering with a molecule's function or lysis of pathogenic cells or pathogens. Recombinant vaccines are another application of recombinant proteins that trigger the body's natural defence immune response to protect against infectious diseases. For example, the recombinant hepatitis virus surface antigen (HBsAg) vaccine can be used to immunise and protect against HBV infection (Akash et al, 2015).

1.5.2.2 Applications in Research

Recombinant proteins are important in the study of many subjects including cell and molecular biology, structural and biophysical studies, immunology and biochemistry. Recombinant bioactive proteins are used to study the function of the protein in different cellular activities, such as metabolism, growth, cell signalling and disease progression, transcription, translation, protein modification, depending on the bioactivity of the proteins (Andersen and Krummen, 2002).

Recombinant proteins and antibodies are useful tools in the understanding of protein-protein interactions. They have been applied in several laboratory techniques, such as ELISA, western blot, and immunohistochemistry (IHC) (Andersen and Krummen, 2002).

1.5.2.3 Protein quality control

Protein quality depends on the evaluation of three important aspects; purity, homogeneity and structural conformity. Purity is the most important aspect, since recombinant proteins are usually contaminated with proteins and other molecules from the host cells, such as nucleic acids, endotoxins and other proteins, or by the extraction and purification procedures (Glase, 1995); and

these contaminants are serious, especially for therapeutic applications (Dullah and Ongkudon, 2016). Heterogeneity is also important for recombinant protein quality, since low or high molecular weight soluble protein aggregates form by the association of two or more polypeptide chains through non-covalent bonds to form dimers and tetramers. These dimers and tetramers affect the protein activity, immune response and structural assays such as crystallization (Murphy and Roberts, 2013). A valuable complementary feature in protein quality control is the validation of the protein folding. Protein conformational changes circular dichroism (CD) is preferred for determination of the folding properties, secondary structures, mutations, and fusion tags effects on the protein stability (Healey et al, 2017; Zvonova et al, 2017).

Hypotheses:

- 1- Based on the current studies and evidence described above, I hypothesised that IL-37a was expressed and induced in immune cells including B cells.
- 2- Based on comparison at the C-terminal with IL-37b, IL-37a should be bioactive with anti-inflammatory effects.
- 3- IL-37b function in innate immune cells as an inflammatory suppressive cytokine, however IL-37a and b isoforms function on B cells is unknown.
- 4- Based on the differences at the N-terminal, IL-37a and b regulate different genes and pathways.

Aims:

1. To produce recombinant IL-37a and b (Chapter 3).
2. To find out the function of IL-37a in B cells (Chapter 4).
4. To explore the differences between IL-37a and b (Chapter 5).

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Mice model

Transgenic IL-37a C57BL/6 mice and wt mice were housed in the Biological Services facility, University of Glasgow, according to United Kingdom Home Office regulation. The mice were used typically starting at 4-6 weeks old. IL-37a and wt mice were kindly supplied by Dr. Xu (Institute of Infection, Immunity and Inflammation. University of Glasgow/ Glasgow, UK).

2.2 Cell stimulators

Cell stimulators are illustrated in table 2.1 with the appropriate concentrations that used.

Table 2.1 Stimulators used in cell activation

cell stimuli	Supplier	working concentrations
Human CD40	Enzo Life Sciences	2ug/ml
Human anti-IgM	Jackson ImmunoResearch	10 ug/ml
Human IL-2	eBioscience	10 ng/ml
Human IL-21	eBioscience	50 ng/ml
Lipofectamine™ RNAiMAX	Thermo Fisher	25 ul/well (96 well plate)
CpG	InvivoGen	3 ug/ml
LPS	Thermo Fisher	100 ng/ml, 10ug/ml
rhIL-37a	House made	50 ng/ml
rhIL-37a	House made	50 pg/ml
rhIL-37b	House made	50 ng/ml
rhIL-37b	House made	50 pg/ml

2.3 Antibodies

Antibodies used for ELISA and western blotting are listed in Table 2.2

Table 2.2 Antibodies used in ELISA and western blotting

Antibody	Produced in	Stock concentration	Dilution	Company: Catalogue number
Anti-6 His-tag	Mouse	0.1 mg/ml	1 :1000	Abcam: ab18184
horseradish peroxidase (HRP)	Rabbit	1.0 mg/ml	1:2000	Abcam: ab97046
Biotinylated anti-human IgG	Mouse	1 mg/ml	1:1000	Invitrogen: 05-4220

2.4 Chemicals and reagents

Chemicals and buffers used throughout the project are listed in Tables 2.3 and 2.4.

Table 2.3 Chemicals

Chemicals	Supplier	Usage
1M /1N hydrochloric acid	Sigma-Aldrich, USA	ELISA
Tween® 20	Sigma-Aldrich, USA	ELISA
3, 3', 5, 5'-Tetramethylbenzidine (TMB)	eBioscience, USA	ELISA
Imidazole	Sigma-Aldrich, USA	Protein purification
EDTA-free complete protease inhibitor cocktail	Sigma-Aldrich, USA	Protein purification
DNAseI	Sigma-Aldrich, USA	Protein purification
Isopropyl β -D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich, USA	Protein purification
30% (v/v) Acrylamide	Sigma-Aldrich, USA	Protein purification
10% (w/v) SDS	Sigma-Aldrich, USA	Protein purification
10% (w/v) Ammonium persulfate (APS)	Sigma-Aldrich, USA	Protein purification
N, N, N', N'-tetramethylethylene-diamine (TEMED)	Sigma-Aldrich, USA	Protein purification
Lysozyme	Sigma-Aldrich, USA	Protein purification
Ampicillin	Sigma-Aldrich, USA	Protein purification
Kanamycin	Sigma-Aldrich, USA	Protein purification

Table 2.4 Buffers

Buffer	Composition
Phosphate buffered saline (PBS)	NaCl (0.137 M), Na ₂ HPO ₄ (0.01 M), KCl (0.0027 M), KH ₂ PO ₄ (0.0018 M), pH7.4
ELISA coating buffer	100 mM NaHCO ₃ pH 8.4, unless specified otherwise
ELISA wash buffer	0.05% (v/v) Tween-20 in PBS pH 7.4, unless specified otherwise
ELISA assay buffer	10% (v/v) FBS in PBS, unless specified otherwise
Nickel binding buffer	10 mM Imidazole, 20 mM Tris-Base, 500 mM NaCl
Nickel gradient buffer (Elution buffer)	500 mM Imidazole, 20 mM Tris-Base, 500 mM NaCl
10X SDS running buffer	248 mM Tris base, 1.92 M glycine, 1% (w/v) SDS , in 2 liter, pH 8.3
Coomassie stain buffer	0.1% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol and 10% (v/v) glacial acetic acid
De-staining solution	30% (v/v) methanol, 10% (v/v) glacial acetic acid
Lura bertani	1% (w/v) NaCl, 1% Trypton (w/v), 0.5% (w/v) yeast , pH 7.5
Tris buffer	250 mM NaCl, 50 mM Tris PH=7.5.
5X SDS-PAGE sample loading buffer	0.25 M Tris-HCl pH 6.8, 0.5 M DTT, 10 % (w/v) SDS, 50 % (v/v) Glycerol, 0.5 % (v/v) bromphenol blue.

2.5 Preparation and transformation of chemically competent *E. coli* cells

In order to purify IL-37a and b, plasmids for each isoform were transformed into competent *E. coli* DH5 α cells. To prepare chemically competent *E. coli*, bacterial cells were cultured overnight in Lura Bertani (LB) medium at 37°C and 120 rpm. 100 ml of fresh LB was inoculated with 1 ml of the overnight culture. The culture was incubated at 37°C and 120 rpm for approximately 1.5-2 hours until OD 600 reached 0.350. The flask was immediately transferred in to on ice for 10 minutes to stop bacterial growth. The culture was split between two 50 ml conical tubes, and both were pelleted at 4°C at 400 xg for 10 minutes. The supernatant was removed and cell pellets re-suspended in 15 ml ice-cold, sterile 0.1 mM CaCl₂ solution before being re-pelleted at 400 Xg, 4°C for 15 minutes. After that, the pellets were re-suspended in 2 ml of 0.1 mM CaCl₂ solution and 200 μ l frozen at -80 °C with 20% (v/v) sterile glycerol. The bacterial cells should be kept on ice at all times. For transformation, aliquots of competent cells were thawed on ice before adding 10 μ l of the ligation mix. The reaction mixture was incubated on ice for 15 minutes before being heat-shocked in a 42°C hot plate for 90 seconds, and then immediately placed on ice. After 2 minutes, 600 μ l of LB liquid medium was added and incubated for (1-2) hours at 37°C on an orbital shaker set at 120 rpm. The transformation mixture was centrifuged at 400 xg for 1 minute. The supernatant was decanted and the pellet re-suspended in the residual mediaby vortexing. Subsequently, transformation mixture was spread on to LB plates supplemented with 100 μ g/ml ampicillin or kanamycin. All plates were incubated at 37°C for 24 hours.

2.5.1 Plasmid DNA extraction

Plasmid DNA was extracted from DH5 α strains of *E. coli* using the QIAprep®Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.5.2 Storage and bacterial growth conditions

All bacteria strains were stored for long term as glycerol stocks held at -80°C. Single colony was grown in the appropriate selection media supplemented with the correct antibiotic and stored in 1ml aliquots with 10% (v/v) glycerol. DH5 α *E. coli* strain was routinely grown at 37°C in typical LB agar or broth. Media were

supplemented with (50 µl/ml or 100 µl/ml) of Ampicillin or Kanamycin antibiotics respectively according to the transfected plasmid.

2.6 Recombinant IL-37 production

The His-tagged recombinant IL-37 proteins were produced in *E.coli* and affinity purified using Nickel-nitrilo triacetic acid-agarose (Ni-NTA) in Dr. Xu's lab.

2.6.1 Recombinant human IL-37a and IL-37b purification

The His-tagged human IL-37 plasmids (pET-28a- IL-37b and pET21 a- IL-37a) for IL-37b and IL-37a respectively were prepared by Dr. Xu's lab, and used for recombinant IL-37 b and IL-37a protein production in *E. coli* using immobilized metal affinity chromatography (IMAC) followed by size exclusion chromatography (SEC).

2.6.2 Recombinant IL-37 test expression

Initially the levels of expression of both recombinant IL-37 proteins were assessed in *E. coli* host strains BL21 (DE3) PlysS competent cells using different time points. Single colony containing recombinant IL-37 vector DNA (pET-28a/IL-37b or pET21 a/IL-37a) was used to inoculate 10 ml of LB broth supplement with 50 µg/ml of kanamycin or 100 µg mlampicillin for IL-37b and IL-37a respectively; and grown overnight at 37 °C with shaking. The following day, 100 µl of overnight culture were added to 5 ml of LB broth with antibiotic and incubated with shaking until an optimum density of 600 (OD 600) reached (0.4-0.6). The protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, Poole, Dorset, UK) directly to the cell suspension. Expression was induced for (1-7) hours at 37 °C with shaking at 180 rpm. Following incubation, 200 µl of broth were collected and centrifuged at 400 xg at 4 °C for 10 minutes. Supernatants were decanted and bacterial pellets were weighed and then stored frozen at -80 °C until protein expression tested on sodium dodecyl sulphate polyacrylamide SDS-PAGE.

2.6.3 Large scale expression of rIL-37 in *E. coli*

For large-scale expression in *E. coli*, a single colony of bacteria containing recombinant vector DNA (pET-28a/IL-37b or pET21 a/IL-37a) was used to inoculate a 60 ml overnight culture as described in the previous section. The following day, the overnight culture was diluted 1/100 in fresh 625 ml of LB broth (6.25 ml for a 625 ml culture) and incubated at 37°C with shaking at 180 rpm until an OD 600 of reached (0.4-0.6). Recombinant IL-37 protein expression was induced with 1 mM of IPTG for (4.5-5.0) hours and the cells were harvested by centrifugation at 400 xg at 4°C for 10 minutes; using the Avanti J-26PI centrifuge (Beckman Coulter, Inc). Supernatants were decanted and the bacterial pellet collected and stored at -80 °C until protein purification. 5 litres of LB broth were used in each experiment.

2.6.4 Purification of recombinant IL-37 from *E. coli* using immobilized metal affinity chromatography (IMAC)

Following recombinant IL-37 expression containing the N-terminal 6X His-tag as described in the previous section, the cell pellet was thawed on ice and re-suspended in 35 ml of lysis buffer (35 ml of binding buffer, 1mg/ml lysozyme, 1M MgCl₂, pH7.9, one tablet of EDTA-free complete protease inhibitor cocktail and 5 µg/ml DNase I). The pellet was mixed into a homogeneous suspension in lysis buffer, and then sonicated 10 cycles with 15 seconds on, and 45 seconds off, keeping on ice throughout. The total cell lysate was centrifuged for 30 minutes at 5000 xg at 4°C in 50 ml conical centrifuge tubes (ThermoFisher Scientific, Loughborough, UK). After centrifugation, the target protein was purified with the Nickel nitrilo triacetic acid (Ni-NTA) column (His-Trap™ HP column, GE Health care), according to the manufacturer's instructions, using the Akta Purifier chromatography system (UPC 10, GE Healthcare). Purification of the target proteins was confirmed by SDS-PAGE, and Coomassie blue staining. The purified protein dialysed in 200 mM NaCl, 50 mM Tris, pH 7.5 overnight at 4 °C, using standard dialysis membrane 5 kDa (Fisher Scientific), and the next day purified by gel filtration.

2.6.5 Purification of rIL-37 using size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) (also known as gel filtration column chromatography), is a method used to separate proteins, primarily based on molecular size. A sample volume of up to 5ml was loaded onto a Sephadex S-75 column (GE Healthcare) according to the manufacturer's protocol. The column was run using the Prime purifier chromatography system (GE Healthcare), and the elute was collected into 1.5 ml/fraction. Samples from the observed peaks were collected and analysed by SDS-PAGE.

2.6.6 Sodium dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to check IL-37 expression and purity, following the steps below:

SDS-PAGE preparation: To prepare 15% SDS-PAGE the following chemicals were mixed: H₂O 1.1 ml, 30% Acrylamide 2.5 ml, 1.5 M Tris-HCl (pH 8.8) 1.3 ml, 10% SDS 50 µl, 10 % (w/v) ammonium persulfate (APS) 50 µl and N, N, N', N'-tetramethylethylenediamine (TEMED) 2 µl. The gel poured to the cast and left approximately 2 cm below the bottom of the comb for the stacking gel. The top of the gel was layered with isopropanol to remove any bubbles and to keep the gel from drying out. After the gel completely polymerized, the isopropanol was removed, and the gel washed with distilled water (dH₂O). The stacking gel was then prepared by mixing H₂O 1.4 ml, 30% Acrylamide 0.33 ml, 1 M Tris-HCl (pH 6.8) 0.25 ml, 10% (w/v) SDS 20 µl, 10% (w/v) APS 20 µl and TEMED 2 µl; the stacking gel was poured on the top of the separation gel; the comb was added to make the wells.

Sample running: The purified recombinant IL-37 was diluted 1:1 with 2X SDS loading buffer composed of (125 mM Tris-HCl, 3% SDS, 40% glycerol and 5% β-mercaptoethanol, pH 6.8), and incubated at 95 °C for 10 minutes before being loaded on to 15% SDS gel. Samples were separated at a constant voltage of 160 V for 1 hour in a 1X running buffer. 5 µl of SeeBlue Plus 2 pre-staining molecular weight standards (Invitrogen, Life Technologies, Paisley, UK) were used for molecular size estimation. Once electrophorised, the gel was stained with Coomassie blue (the next section).

2.6.6.1 Coomassie blue staining of proteins

Within this study Coomassie blue dye was used to visualise proteins separated by SDS-PAGE. Following electrophoresis, the gel was incubated at room temperature with constant shaking in a solution of Coomassie brilliant blue stain (0.1% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol and 10% (v/v) glacial acetic acid) for 30 minutes. The gel was then washed with a de-staining solution (10% methanol, 10% glacial acetic acid) at room temperature with constant shaking for 10 minutes. This washing step was repeated several times to enable the visualisation of individual protein bands.

2.7 Western blotting

Different fractions of the purified proteins (rIL-37a and rIL-37b) after Ni-NTA purification were electrophoretically separated for 1 hour (150 V). The separated proteins were transferred on to a nitrocellulose membrane for 1 hour (30 V) in an X Cell II blot module (Invitrogen, UK) containing transfer buffer. The membrane was then washed in PBS, and blocked with 10 % (w/v) non-fat milk powder in PBS for 1 hour at room temperature. After 3 × 5 minutes washes in PBS, the membrane was probed with primary antibody mouse anti-6His-tag (1:1000 dilution) in 5% (w/v) non-fat milk powder in PBS-tween for 2 hours at room temperature. The membrane was washed in 3×5 minutes washes in PBS-tween; and probed with horseradish peroxidase-conjugated anti mouse secondary antibody (1:2000) in PBS-Tween for 1 hour at room temperature. After the 3×5 minutes washes in PBS, the membrane was incubated in chemiluminescent substrate (Amersham ECL, Amersham Bioscience) for 30 seconds. The membrane was then drained from the excess substrate, and imaged with the fluorescence scanner.

2.8 Protein concentration measurement

Recombinant human IL-37 protein concentrations were determined using Coomassie Protein Assay Kit (Bradford). First, 2-fold serial dilutions of the standard protein (Bovine serum albumin standards, Thermo Scientific, USA) were performed. 480 µl of Coomassie Reagent were added to 20 µl of the serial diluted standards, samples and PBS as blank, mixed well and incubated 10 minutes at room temperature. 150 µl of the samples were transferred to a 96-

well flat-bottom plate; the OD values were measured using an ELISA reader using 600-nm absorbance. The protein concentrations in the samples were determined by interpolating the readings to the OD values of the standards.

2.9 Endotoxin removal

Endotoxin in the purified recombinant human IL-37a and b (rIL-37a, rIL-37b) proteins was removed by purification with polymyxin B columns following the manufacturer's instruction. A new column (EndoTrap) was placed in a holder and the top cap removed first then the bottom cap, allowing the storage solution to drain from the column (approximately 8 minutes), making sure that the EndoTrap resin does not run dry. The column was activated as follows: the column was filled with regeneration buffer (approximately 3 ml) and allowed to drain out, repeated once; the column was filled with equilibration buffer (approximately 3 ml) and drained out, repeated once. Then the column was filled with sample (purified rhIL-37 proteins) in PBS and the flow through liquid collected immediately; the column could be repeatedly filled with up to 50 ml of samples. The column was filled with 1 ml of PBS and drained out to elute samples completely.

2.9.1 Endotoxin detection

Endotoxin levels in the rhIL-37 preparations were measured using Limulusamebocyte lysate (LAL) QCL-1000 pyrogen test following the manufacturer's manual. The microplate was preheated to 37°C in a heating block adapter (Thermo Fisher Scientific, USA). While leaving the plate on the heating block, 50 µl of standards and samples were added to the wells, for the blank control; 50 µl of LAL reagent water was used. 50 µl of LAL were then added into each well using a multi-channel pipette in the order of the reagent addition. The plate was tapped for mixing and incubated for 10 minutes followed by adding 100 µl of preheated substrate solution into each well. 16 minutes later, the reaction was stopped by adding 100 µl of stop reagent in the same sequence as the previous steps. The plate was removed from the heat block and read with an ELISA reader (Dynex Technologies, USA) using absorbance at 410 nm, the optical density (OD) recorded and the samples' concentration calculated based on the linear standard curve obtained by the standards on the same plate.

2.10 Bioactivity test of recombinant IL-37a and b in RAW 264.7 cell line.

To determine the bioactivity of the in-house prepared rIL-37a and b, murine RAW 264.7 cells (10^6 /ml per well) were cultured in 24 well plate using DMEM medium, stimulated with LPS (100 ng/ml) and treated with different doses of rIL-37a and rIL-37b purified by different columns previously. Cells were harvested after 24 hours of culturing for gene expression estimation; the culture supernatant was tested for IL-6 ELISA using murine IL-6 cytokine ELISA kit from R&D system, and the cells pellet were harvested for gene expression by qPCR.

RAW 264.7 cells were cultured in a 75 cm² flask with total volume of 10 ml DMEM medium. Cells splitting was carried out after confluence, which was estimated to be around 60-70%. The growth of RAW cells was monitored; once a monolayer of cells had partially covered the flask (approximately 70% confluence), cells were removed by lifting, and passaged as follows. RAW 264.7 cells are quite adherent to tissue culture flask; thus, trypsin does not lift the cells; therefore, cells were washed twice with 3 ml of PBS, gently lifted and scraped off the flask with a scraper and warm media. The cell suspension was collected from the flask; spun for 8 minutes at 300 xg; re-suspended into fresh media; seeded in to a new flask with 1:3, 1:4 or 1:5 dilutions; and placed back into humidified incubator (5% CO₂ at 37°C).

2.11 Protein Crystallization

Purified IL-37b was dialysed overnight against PBS pH 7.5, then concentrated into 14 mg/ml using Sartorius viva spin (50 ml tube from thermos Fisher Company) and kept on ice. Several commercial High throughput screens were used including: JCSG- plus-HT-96, MIDAS-96, Morpheus-HT-96, and PACT premier-HT-96 using drop vapour diffusion. All crystal trays were set up using a Cartesian Honeybee robot (Hamilton, UK). Crystal drop was set up 1:1 protein: buffer ratio, with a final volume of 1 ul. The trays were kept at 17 °C in the incubator.

2.12 Mass Spectroscopy (MS)

IL-37a band on the SDS-PAGE was cut, and put in an epindroph, sent to Wolfson Wohl Cancer Research Centre/ University of Glasgow, for confirming IL-37a identity.

2.13 Circular Dichroism (CD)

IL-37b dialysed in PBS, and CD performed at final concentration of 120 ug/ml, at Institute of Molecular Cell & Systems Biology/ Glasgow University

2.14 Tissue culture

Cells were cultured in complete medium (Roswell Park Memorial Institute (RPMI) 1640 for suspension cells or Dulbecco's modification of Eagle medium (DMEM) for adherent cells, supplemented with 10% heat inactivated FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2 mM L-glutamine (Invitrogen Ltd., UK, unless otherwise specified). Cultures were incubated at 37.0°C in a humidified incubator (Heraeus Instruments, Germany) supplemented with 5% CO₂. Viable cells were counted with a Neubauer haemocytometer (Weber Scientific International Ltd, UK) on a Nikon Labphot microscope, staining with 0.1% (w/v) Trypan Blue (Sigma-Aldrich, USA).

2.14.1 THP-1 cells line

Human monocytic THP-1 cells were used to identify IL-37 isoform expression. THP-1 cells were cultured in 24 well plate (10⁶/ml) using RPMI medium, cells were stimulated with different doses of LPS (100 and 200 ng/ml), and IL-37 isoforms expression estimated by qPCR. After determination of the suitable dose of LPS for IL-37 isoforms expression, different time points (3, 16, and 24 hours) were used to determine the best time of IL-37 expression.

2.14.1.1 IL-37 knocks down in THP-1 cells

On the day of transfection, cells were harvested and seeded to 24 well plate at the density of 1.5 x 10⁵ cells/ ml/ well in a culture medium without antibiotics and left for an hour in the incubator. 100 nM siRNA (Thermo Fisher Scientific) and Lipofectamine RNAiMax reagent (Thermo Fisher Scientific) were diluted as

described in the protocol and incubated for 5 minutes at room temperature. A mixture of primers was used to silence the isoforms, and the sequences of the primers used can be found in table 2.5. The complex formed was gently mixed with the cells and incubated for 48 hours. Control cells were transfected with negative control siRNA (Silencer® select negative control No.1 siRNA, Thermo Fisher Scientific). Following the incubation, cells were pelleted by centrifugation at 200 xg for 5 minutes; re-suspended to fresh complete media with antibiotics and centrifuged again to maximise the removal of the lipid complex. Cells were seeded again to the 24 well plate and incubated for an hour in the incubator to settle down before the LPS stimulation. Cells were stimulated with 20 ng/ml of LPS for 6 hours, harvested and stored at -20°C until further analysis. The only difference in control cells was absence of LPS stimulation.

Table 2.5 Small interference RNA siRNA oligonucleotides

siRNA IL-37a	Target 16-38 nucleotides	sense 5'- GAGGGAAACAGAAACCAAAUU-3'
		antisense 5'- UUUGGUUUCUGUUUCCCUCUU-3'
	Target 35-57 nucleotides	sense 5'- GGAAAGAACAGCUUUAAGAUU-3'
		antisense 5'-UCUUAAAAGCUGUUCUUUCCUU-3'
siRNA IL-37	Target conserved regions of all isoforms	sense 5'-UCAAGGAUGAGGCUAAUGCUU-3'
		antisense 5'-CAAUGUGUUCCUGUUCUCUU-3'
		sense 5'-UUACAAUUGCAGGAGGUGCUU-3'
		antisense 5'-UUAUCCUUGUCACAGUAGAUU-3'

2.14.2 3T3-ms CD40L feeder cell preparation

3T3-ms CD40L cells were cultured in complete DMEM (10% heat inactivated FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2 mM L-glutamine). 1×10^6 3T3-msCD40L cells were seeded in T-75 cell culture flasks containing 15-20 ml of complete DMEM medium. When cells reached (80-90)% confluency, the culture medium was aspirated, the cells washed with 10 ml of PBS, trypsinized by adding 4 ml of trypsin and incubated at 37°C for 4 minutes, and digestion stopped by adding 5 ml of whole medium that contains serum anti-trypsin. The cells were harvested into 50-ml conical tubes, centrifuged at 300 xg for 10 minutes, re-suspended the cells at a concentration of between (5-10) $\times 10^6$ cells/ml and irradiated at 5000 rads using an XRAD225 x-ray irradiation system (Precision X-ray Inc).

2.15 Murine CD19⁺ B cell

2.15.1 Murine CD19⁺ B cell isolation from Spleenocytes

The wt and IL-37a-tg mice were sacrificed at approximately 4-6 weeks of age. Spleens were isolated and kept in PBS on ice until cells extraction. The spleen was placed into a 70 μ M cell strainer, which in turn was placed in an appropriate petri dish with 4 ml of medium were added to it, and the spleen gently was mashed. The strainer was washed with media, and the cells collected in a 50 ml Corning tube with extra 10 ml medium, spun at 300 xg for 5 minutes at +4 °C. The medium discarded; and 3 ml of pre-warmed lysing buffer added, and the mixture incubated on ice for 5 minutes to lyse red blood cells. Following the incubation, 40 ml of cold 1X PBS were added, and spun at 300 xg for 5 minutes at +40 °C; the PBS discarded and the cells pellet re-suspended in 5 ml of media and mix well; keep on ice until proceeding to Magnetic activated cell sorting (MACS) separation.

Using murine CD19 magnetic micro beads, B cells were separated from the spleenocytes by following the standard manufacturer's instructions. Spleenocytes were washed with cold MACS buffer (PBS pH 7.2, 0.5% (v/v) bovine serum albumin, and 2 mM EDTA); centrifuged at 300 xg for 10 minutes; and the buffer was aspirated; the cells counted and re-suspended in 90 μ l of MACS buffer per 10^7 cells with addition of 10 μ l of CD19 micro beads per total 10^7 cells; mixed well and incubated in the refrigerator for 15 minutes at +40 °C. After that, cells were washed with 2ml MACS buffer per 10^7 cells; centrifuged for 10 minutes at 300 xg; re-suspended in 500 μ l; mixed well; applied to LS separation column that was placed in an appropriate magnetic field of the suitable MACS separator rinsed previously with 3 ml of MACS buffer. The unlabelled cells were collected from the column and the column washed three times with 3 ml of MACS buffer. The column was removed from the magnetic separator and placed on a suitable tube; 5 ml of MACS buffer were added to the column and the labelled cells flushed out by firmly pushing the plunger into the column.

2.15.2 Primary culture of murine CD19⁺ B cells

Murine CD19⁺ B cells that had been separated previously by MACS technique, cells were cultured in PRMI 1640 containing 100 units/ml penicillin, 100 µg/ml streptomycin.

2.15.2.1 Murine CD19⁺ B cells

Murine CD19⁺ B cells cultured in complete PRMI 1640 medium at concentration of (10⁶ cells/ml), cells were stimulated with different stimuli (LPS (10 µg/ml), 3T3 cells (15 x 10⁴ cells/ml), rIL37a (50 ng/ml), rIL-37b (50 ng/ml) in 24 well plate for different time according to the experiment condition. The supernatant collected for antibody estimation using ELISA technique; and the cells harvested for gene expression by qPCR

2.16 Human CD19⁺B cell isolation

To isolate B cells, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat and purified by standard manufacturing procedures using Histopaque-1077 (Sigma, USA). Heparinized venous blood was collected from the healthy donors, the collected blood diluted 1:1 with DPBS 3 ml of Histopaque added to the 15 ml centrifuge tube and 10 ml of diluted blood carefully layered. After centrifuging, at 400 xg for 30 minutes at (18-20) °C, the upper layer (plasma) was aspirated and the lymphocytes layer (opaque interface) then removed to new centrifuge tube using sterile Pasteur pipette. Cells were washed three times in DPBS and spun at 250 xg for 10 minutes at 18-20°C; the supernatant poured off, and this step repeated at least 2 times. B cells were separated from PBMC using CD19 magnetic micro beads, keeping cells and buffer cold. Following the manufacturer's instructions; cells were counted, washed with 1-2 ml of MACS buffer (PBS pH 7.2, 0.5% bovine serum albumin, and 2 mM EDTA) per 10⁷ PBMC cells and centrifuged at 300 xg for 10 minutes. The buffer aspirated and the cells re-suspended in MACS buffer (80 µl per 10⁷ cells); with addition of 20 µl CD19 micro beads, the cells and the CD19 beads mixed well; incubated in the refrigerator for 15 minutes; washed after incubation with 1-2 ml of MACS buffer per 10⁷, centrifuged for 10 minutes at 300 xg. The cells re-suspended in 500 µl MACS buffer per 10⁸ cells; mixed well; and applied to LS separation column (Miltenyi Biotech) placed in a magnetic field rinsed previously

with 3 ml of MACS buffer. The unlabelled cells were collected from the column; and the labelled cells on the column washed three times with 3 ml/each time. The column removed from the magnetic field and placed quickly on a 15 ml tube; 5 ml of cold buffer was then added to the column and the labelled cells flushed out by firmly pushing the plunger into the column.

2.16.1 Primary culture of human B cells

Human B cells, which were separated by MACS, cultured in RPMI 1640 containing 100 units/ml penicillin, 100 µg/ml streptomycin in 24 well plate (10^6 cells/ml).

2.16.1.1 Human CD19⁺B cells stimulation

CD19⁺ B cells cultured in complete RPMI medium, stimulated with different stimulators including: (CPG (3 µg/ml), 3T3 cells (15×10^4 cells/ml), CD40 Ligand (2 ng/ml), anti-IgM (10 µg/ml), IL-21 (50 ng/ml), IL-2 (10 ng/ml), rIL37a (50 pg/ml and 50 ng/ml), and rIL-37b (50 pg/ml & 50 ng/ml), and cells incubated for different periods of time according to the experiment condition. Cells supernatant collected for IgG determination by ELISA; and cells pellet harvested for gene expression by qPCR.

2.17 Gene expression assay

2.17.1 Quantitative-PCR analysis

2.17.1.1 Extraction of total RNA from cells

A spin-column based RNA extraction kit (Qiagen, Germany) was used for this purpose. All procedures were conducted according to manufacturer's instruction manuals. Briefly, add 350 µl of Buffer RLT to lyse cells up to 5×10^6 , then transfer the lysate directly into a QIA shredder spin column placed in a 2 ml collection tube; and centrifuge for 2 minutes at 6500 xg. Transfer flow-through to an RNeasy spin column placed in a 2 ml collection tube, centrifuge for 15 seconds at 4000 xg. Discard the flow-through; add 700 µl Buffer RW1 to RNeasy spin column, centrifuge for 15 seconds at 4000 xg, discard the flow-through; wash RNeasy spin column twice with 500 µl Buffer RPE, centrifuge at 4000 xg rpm for 15 seconds the first time and 2 minutes the second time. Discard the flow-through; place the RNeasy spin column in a new 1.5 ml collection tube, add

40 µl RNase free water directly to RNeasy spin column membrane, centrifuge for 1 minute at 4000 xg to elute the RNA.

2.17.1.2 Reverse Transcription

Reverse Transcription (RT) of RNA into cDNA was carried out using High-Capacity cDNA Reverse Transcription Kits according to manufacturer's protocol (Applied Biosystems, US). Dilute RNA samples to 1.5 µg per 10 µl using nuclease-free water. Prepare 2X reverse transcription master mix, each 10 µl mix containing 2.0 µl of 10X RT Buffer, 0.8 µl of 25X dNTP Mix, 2.0 µl of 10 RT Random Primers, 1.0 µl of MultiScribe reverse transcriptase and 4.2 µl of nuclease-free water. For a 20 µl RT reaction; mix 10 µl of 2X reverse transcription master mix with 10 µl of RNA samples in individual PCR tube, briefly centrifuge the tubes to eliminate any air bubbles; load the tubes in thermal cycler (Eppendorf, UK). Perform reverse transcription reaction in the following conditions: 25 °C for 10 minutes, 37 °C 120 minutes, 85°C for 5 seconds and 4°C to stop the reaction. The cDNA samples were kept at -20 °C until further experiments.

2.17.1.3 Real-time PCR

Real-time polymerase chain reaction (RT-PCR) was performed using Fast SYBR Green master mix (Applied Biosystems, USA): pipette 10 µl of 2X Fast SYBR Green master mix, 1 µl of each forward and reverse PCR primers (Table 2.7). 2 µl of cDNA samples, and 7 µl of nuclease-free water into each well of a 96-well reaction plate; briefly centrifuge the plate to eliminate any air bubbles; load the plate into an Applied Biosystems real-time quantitative PCR instrument; set the thermal cycling condition: 95 °C 20 seconds; 95 °C 3 seconds, 60 °C 30 seconds, repeat 40 cycles; calculate the results using $\Delta\Delta CT$ method (Livak and Schmittgen, 2001, Giulietti et al., 2001). The primers (Integrated DNA Technologies, USA) were synthesized according to the sequences in Tables 2.6 and 2.7.

Table 2.6 Real-time PCR primers

Human	Forward	Reverse
GAPDH	5'- GAGCCACATCGCTCAGACAC -3'	5'- CATGTAGTTGAGGTCAATGAAGG -3'
IL-1F7a	5'- GGGAAACAGAAACCAAAGGA -3'	5'- CCCAGAGTCCAGGACCAGTA -3'
IL-1F7b	5'- ATGTCCTTTGTGGGGGAGAACTCAG - 3'	5'- TGCTATGAGATTCCCAGAGTCCAGGACC -3'
IL-1F7c	5'- CCCCACCATGAATTTTGTTT -3'	5'- CCTTTAGAGACCCCCAGGAG -3'
IL-1F7d	5'- AACCCCAAGTGCTGCTTAGAA -3'	5'- CCCAGAGTCCAGGACCAGTA -3'
IL-1F7e	5'- AACCCCAAGTGCTGCTTAGAA -3'	5'- CCTTTAGAGACCCCCAGGAG -3'
IL-18R1	5'- CCCTGGGCAAAATCTCCACAGCA -3'	5'- ACGCCGAGTTGAAGATCAGGGGT -3'
SIGIRR	5'- GTACCAGAGCAGCACGTTGA -3'	5'- TCAGTGGCTCTGAACTGCAC -3'
TNFA	5'- CAGGGCAATGATCCCAAAGT -3'	5'- TCAATCGGCCCCGACTATCTC -3'
IL10	5'-TCCTCCTGCTTGTCATAGTC -3'	5'-CACCCATTTCTTGAAGTCTCC -3'
IFNB	5'-GCGACACTGTTCTGTTGTC -3'	5'-GCCTCCCATTTCAATTGCCAC -3'
IFNG	5'-TCA GCT CTG CAT CGT TTT GG -3'	5'-GTT CCA TTA TCC GCT AACTGAA -3'
IL12A	5'-TTCGCTTTCATTTTGGGCCG -3'	5'-ATCAGCTTCTCGGTGACACG -3'
IL13	5'-AACCAGAAGGCCTCCGCTCTGC -3'	5'-GGCACTGCAGCCTGACACGTT -3'
IL1A	5'-ACTGCCCAAGATGAAGACCA -3'	5'-CCGTGAGTTTCCCAGAAGAA -3'
IL6	5'-TGCAATAACCACCCCTGACC -3'	5'-AGCTGCGCAGAATGAGATGA -3'
TGFβ	5'-TACCTGAACCCGTGTTGCTCTC -3'	5'-GTTGCTGAGGTATCGCCAGGAA -3'

Table 2.7 Real-time PCR primers

Murine	Forward	Revers
IL-1F7a	5'- GGGAAACAGAAACCAAAGGA -3'	5'- CCCAGAGTCCAGGACCAGTA -3'
Tgfb1	5'-CACCGGAGAGCCCTGGATA -3'	5'-TGTACAGCTGCCGCACACA -3'
Sigirr	5'-GGTGGCAGTCGTGACTCAG -3'	5'-CTGGCTCCCTGTAATGG -3'
Il18r1	5'-CTTCGTCTTGGTGAGAAAAGAAATA -3'	5'-TGTCACAGCAGCATATTACCTG -3'
Gapdh	5'-GGCATGGACTG GGTCAT GAG -3'	5'-TGC ACC ACC AAC TGCTAGC -3'
IFN γ	5'-TTCTTCAGCAACAGCAAGGC -3'	5'-TCAGCAGCGACTCCTTTTCC -3'
Il10	5'-AAC AAA GGA CCA GCT GGA CAAC -3'	5'-CTT GAT TTC TGG GCC ATG CTT -3'
Il13	5'-GCAGCATGGTATGGAGTGTG -3'	5'-TGGCGAAACAGTTGCTTTGT -3'
Tnfsf13 (APRIL)	5'-CAGTCCTGCATCTTGTTCCA -3'	5'-GCAGATAAATTCCAGTGTCCC -3'
Inpp5d (SHIP)	5'- ACTCTGCGTGCTGTTCCGGA -3'	5'-CCTTCGGATGCCTGAACAGTG -3'
Lyn	5'CTATCCCGTCGCGTGAAC -3'	5'-CATCCCATATTTCTCGCTCGT -3'

2.18 Measurement of B cell proliferation

The measurement of DNA replication determined by the incorporation of 5-bromo-2-deoxyuridine (BrdU) (Roche, UK) was used to measure the cellular proliferation. Briefly human B cells were grown in 96 -well flat bottom plates (10^5 cells/well) for 3, 4, 7 days; and the cellular proliferation was determined by incorporation of BrdU. 0.1 μ l of BrdU labling reagent (10 mM 5-bromo-2-deoxyuridine in PBS, pH 7.4) was added per well for a 96 well plate for B cells cultured 2, 3, 6 days before the end of the experiments. After 24 hours of BrdU addition, the plate was centrifuged at 300 \times g for 10 minutes, and the medium removed by flicking off or aspiration; and following the same step, the cells were washed with 150 μ l of cold PBS added by multi-channel pipette to remove the excess of BrdU reagent. The plate dried in the incubator at 37 °C without CO₂. Next, 200 μ l of the FixDenat reagent were added to each well; incubated at room temperature for 2 hours; then removed by flicking off and tapping; followed by addition of 100 μ l of anti-BrdU-POD solution incubated for 3 hours. Finally, the anti-BrdU-POD solution was removed by flicking off; the wells washed with 300 μ l of PBS; 100 μ l of the substrate added per well; incubated for 5-20 minutes until colour development. 25 μ l of the stop solution (1 M H₂SO₄) per well added, and the plate read with ELISA reader at 450 nm after 1 minute shaking at 200 \times g

2.19 Enzyme linked immunoassay (ELISA)

Murine IL-6 cytokine and IgG isotype were analysed by ELISA, using IL-6 ELISA kit (R&D system) and mouse IgG kit (affymetrix, eBioscience). All mice ELISA experiments were conducted according to the manufacturer's protocol.

- 1- *Murine IgG*: The ELISA plate (Costar 9018, Corning, USA) was coated with 100 μ l of capture antibody diluted (1:250) in 1X coating buffer (1X PBS in deionised water); the plate sealed and incubated in the fridge over-night at 4° C. The wells were aspirated, washed twice with 400 μ l/well with wash buffer (1X PBS, 0.05% Tween-20); and the plate blocked with 250 μ l/well assay diluent 2X (1X PBS, 10% BSA, 1% tween-20) that supplemented the kit, and incubated at room temperature for 2 hours, washing as step 2. 2 fold serial dilutions in 1X blocking buffer of the standard were prepared for the

standard curve. The cell culture samples were pre-diluted 2X in 1X blocking buffer in triplicate wells; followed by addition of 50 μ l of the detection antibody to all wells; the plate covered, sealed and incubated at room temperature for 3 hours on a microplate shaker at 200 xg. The wells were aspirated and the plate washed as step 2 for total wash of 4 times. 100 μ l of the substrate solution added and incubated for 15 minutes; followed by the addition of 100 μ l of stop solution to each well; and the plate read at 450 nm and the values of 570 subtracted from those of 450 nm; data were analysed using Excel.

- 2- Human IgG isotype levels: ELISA 96 well plate (Costar 9018, Corning, USA) was coated with 100 μ l/well of cells supernatant, and 100 μ l per well of 2-fold serial dilutions of the top standard IgG, added to make the standard curve and blanks (PBS buffer or media, respectively) as control. The plate was incubated at 4°C overnight. After incubation, the plate was washed 4 times with > 300 μ l/well wash buffer (1X PBS, 0.05% tween-20), blotted on absorbent paper to remove any residual buffer; and the wells blocked with 200 μ l/well assay diluent (1X PBS, 10% FBS), incubated over-night at 4°C and washed as step 2. Then, 100 μ l/well of Biotin-conjugated detection antibody diluted in Assay diluents were added and incubated at room temperature for 1 hour. Washing as previous steps. 100 μ l per well of Avidin-HRP diluted in assay diluent and incubated at room temperature for 30 minutes. The plate was washed for a total wash of 7 times. The 100 μ l of Substrate Solution (Tetramethylbenzidine Substrate Solution) were added to each well and incubated at room temperature for 15 minutes. The reaction was stopped by adding 100 μ l per well of Stop Solution (1 M HCl). Plates were read using an ELISA reader (Tecan, Switzerland) at 450 nm and the value read at 570 nm subtracted. The OD was measured against the reagent blanks, standards and test samples, and obtained the relative sample concentration by interpolating the readings to the Standard Curve.

2.20 Next generation sequencing

2.20.1 Microarray

Murine spleenocytes cells were purified and cultured in RPMI, stimulated with 500 ng/ml of LPS for 4 hours, cells harvested and RNA extracted, converted to cDNA, and microarray performed in China by Dr.Xu lab.

Normalization was achieved after importing the Output_fused. The data was statistically analyzed using one way ANOVA. False discovery rate given %5 false genes as it was set to the p-value 0.05 followed by applying multiple correction method using a Benjamini and Hochberg False Discovery Rate. Bio Venn programme used to discriminate each group of genes by Biovenn programme (Elure figures), each group of genes compared with innate immune genes, using IDB web site, and KEGG analysis pathways enrichment

2.21 Statistical analyses

All statistical analyses were performed with Excel software. Different analysis strategies were used depending on the data being investigated. Depending on the normal or skewed distribution of the data, either a T-test or a Mann-Whitney test was used respectively when comparing two sets of data. A one-way ANOVA (or non-parametric equivalent) was used for the comparison of three or more groups. Figure legends state the statistical test used for the analysis of each data set. *P* values of < 0.05 were considered to be statistically significant and illustrated in graphs as * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Data in graphs are shown as mean \pm SD.

Chapter 3

Production, purification and Crystallization of recombinant human IL-37a and IL-37b

3 Production, purification and Crystallization of recombinant human IL-37a and IL-37b

3.1 Introduction

IL-37 gene encodes five-transcript variants (IL-37a-e) due to alternative RNA splicing. While the function of IL-37b has been widely studied and identified as an important immune regulatory cytokine, the functions of the other IL-37 isoforms are largely unknown. The focus of this study is the IL-37a isoform, which was compared with IL-37b. The basis for this was that (1) our preliminary results showed that IL-37a expression is highly inducible in human macrophages by inflammatory stimuli, compared to other IL-37 isoforms (Chapter 4, Fig 4.2). This suggests that IL-37a may also play an important role in the inflammatory response; (2) IL-37a has an N-terminal region that is not homologous to IL-37b, suggesting that IL-37a may have unique biochemical features and functions.

The pro or full length IL-37b is the longest transcript variant among the IL-37 isoforms and is composed of 218 amino acids. It contains caspase-1 cleavage sites at its N-terminus for maturation (Fig 3.1) (Kumar et al, 2002; Sharma et al, 2008; Boraschi et al, 2011; Afonina et al, 2015). The C-terminal of mature protein IL-37b consists of 12 β -strands forming the β -trefoil structure, which is characteristic of IL-1 family cytokines (Fig 3.1). This domain is responsible for these cytokines in order to bind to their receptor and trigger signal transduction (Boraschi et al, 2011; Zhao et al, 2018). IL-37a is shorter than IL-37b and is composed of 192 amino acids (Kumar et al, 2000; Boraschi et al 2011). It has a unique N-terminal region containing a putative nuclear location sequence (NLS) for protein translocation into the nucleus (Taylor et al, 2002; Boraschi et al, 2011). A putative elastase cleavage site down stream of the NLS has also been identified (Boraschi et al, 2011; Abulkhir et al, 2016). This specific N-terminal region may offer IL-37a unique biochemical features, cellular location and biological function. Like other IL-1 family members, IL-37a also possesses the 12 β -strand forming β trefoil domain. This suggests that IL-37a and IL-37b bind to the same receptor for signalling.

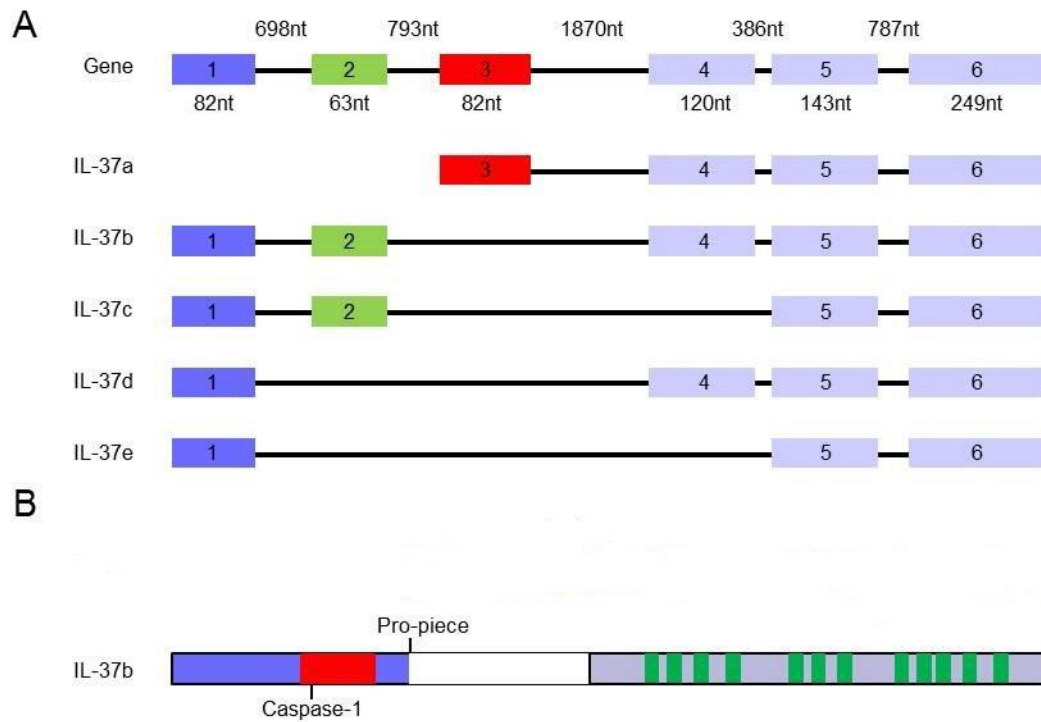


Figure 3.1 The key sites and domains of IL-F7a and IL-1F7b pro-protein.

IL1F7 gene has six exons. A) represents the IL1F7 gene and the five isoforms; the coloured boxes are the exons with their numbers, and the size of each exon is indicated just below the exon boxes; while the size of the intervening introns are at the top of each intron. B) Represents IL-1F7b or IL-37b isoform structure, and illustrates the caspase-1 cleavage site in exon 1, followed by the pro peptide domain; the rests are exons 2, 4, 5, and 6.

Cytokines produced recombinantly in bacteria can be a valuable tool for studying human protein function although these recombinant proteins will lack post-translational modifications such as glycosylation that may be present in the human derived protein. FL and mature human IL-37b proteins have been produced in *E. coli* by several laboratories and companies (Gu et al, 2015; Li et al, 2015). IL-37b constructs have been expressed both in a soluble form in the *E. coli* cytoplasm and in inclusion bodies (Carrió and Villaverde, 2001; Yang et al, 2011; Singh et al, 2015).

In most reports, the recombinant protein has first been purified by nickel affinity chromatography, using Ni-affinity chromatography, followed by gel-filtration chromatography (Stuehr et al, 1991; Harood, 1996; Kaur and Reinhardt, 2012). Ni-affinity chromatography is one of the immobilized, metal affinity chromatography (IMAC) approaches for efficient purification of engineered proteins that carry polyhistidine-tags. The histidine-tags bind to the divalent metal (Ni^{+2} , CO^{+2} or Cu^{+2}) attached to the agarose resin and can be eluted by adding imidazole (Mooney et al, 2014).

Contaminating endotoxins from *E.coli* can be removed from recombinant proteins using different techniques including EndoTrap® affinity chromatography (Guillou et al, 2015). IL-37b purified by this method is bioactive, and can suppress inflammatory responses in vitro and in disease models in vivo (Gu et al, 2015). Furthermore, both FL and mature IL-37b are bioactive and share similar bioactivity in vitro (Kumar et al, 2002). Since bacteria cannot provide the correct glycosylation of human protein this also suggests that glycosylation of IL-37 is not required for human IL-37b bioactivity. However, it is still unknown if IL-37a can be produced in a similar way in *E. coli* and whether IL-37a produced in this way is bioactive.

Hypothesis: The difference in N-terminal sequence between IL-37a and IL-37b influences the structural and biochemical features of these isoforms.

The aim of the work in this chapter was to take the first steps to understand the biological function, structure and importance of IL-37a in biology and in disease. To achieve this, recombinant IL-37a and IL-37b were produced and differences between the IL-37 isoforms in terms of protein structure and biochemical features were studied. This was achieved by:

- 1- Expression of human recombinant IL-37a and IL-37b protein in *E. coli*.
- 2- The development of a purification protocol for IL-37a and IL-37b proteins using multiple chromatography techniques.
- 3- Structural studies using crystallization.

3.2 Results

3.2.1 Differences between IL-37a and IL-37b at the amino acid level and biochemical parameters.

Understanding of the difference between the two IL-37 isoforms at the amino acid level can provide important information for protein purification and for assessing protein stability and solubility. The differences of the two isoforms at the amino acid level were analysed using ProtParam from ExPASy (extensible and integrative portal accessing (Boraschi et al, 2011)). The differences at amino acid level between IL-37a and IL-37b isoforms showed in Tables (3.1) and (3.2).

IL-37a has 9 positively charged arginine and 22 lysine residues. Together they comprise 16.2% of the total protein, while isoform b has 5 arginine and 20 lysine (11.5%). Levels of negatively charged amino acids (glutamate and aspartate) are higher in IL-37b than IL-37a; 12.9% in IL-37b and 11.5% in IL-37a. There is no marked difference between IL-37a and IL-37b protein in the percentage of aliphatic amino acids (Table 3.2). Consequently, there is a clear difference between the two isoforms in their isoelectric point (pI) 9.23 and 6.09 for IL-37a and IL-37b, respectively.

3.2.2 Production of recombinant IL-37b

In order to investigate the structure and biological function of IL-37a, large quantities of recombinant protein may be required. To determine whether IL-37a and IL-37b are different in bioactivity, FL-IL-37a and IL-37b were produced in *E. coli*. The strategy was used to produce recombinant human IL-37a and IL-37b is shown in (Fig 3.2).

Table 3.1 Amino acid composition of human IL-37a and IL37b

Amino acids	IL-37a		IL-37b	
	Number of amino acids	Percentage of amino acids %	Number of amino acids	Percentage of amino acids %
Ala (A)	12	6.2	13	6
Arg (R)	9	4.7	5	2.3
Asn (N)	9	4.7	10	4.6
Asp (D)	9	4.7	11	5
Cys (C)	7	3.6	8	3.7
Gln (Q)	6	3.1	7	3.2
Glu (E)	13	6.8	17	7.8
Gly (G)	11	5.7	13	6
His (H)	5	2.6	6	2.8
Ile (I)	8	4.2	8	3.7
Leu (L)	15	7.8	17	7.8
Lys (K)	22	11.5	20	9.2
Met (M)	4	2.1	6	2.8
Phe (F)	11	5.7	12	5.5
Pro (P)	11	5.7	17	7.8
Ser (S)	21	10.9	25	11.5
Thr (T)	4	2.1	4	1.8
Trp (W)	2	1	3	1.4
Tyr (Y)	3	1.6	3	1.4
Val (V)	10	5.2	13	6

(Gasteiger et al, 2005)

(<https://web.expasy.org/protparam>)

Table 3.2 Parameters for human IL-37a and IL-37b based on their primary sequence

Parameter	IL-37a	IL-37b
Molecular weight (kDa) ^a	21.5	24.1
Theoretical iso electric point (pI)	9.2	6.1
Number of amino Acids	192	218
negatively charged amino acids %	11.5	12.9
positively charged amino acids %	16.2	11.5
Nonpolar aliphatic amino acids %	34.8	33.6
Polar amino acids %	27.6	28.9
Aromatic amino acids %	8.4	8.3

a: kilo Dalton

(Gasteiger et al, 2005).

(Compute pI/Mw. http://web.expasy.org/compute_pi)

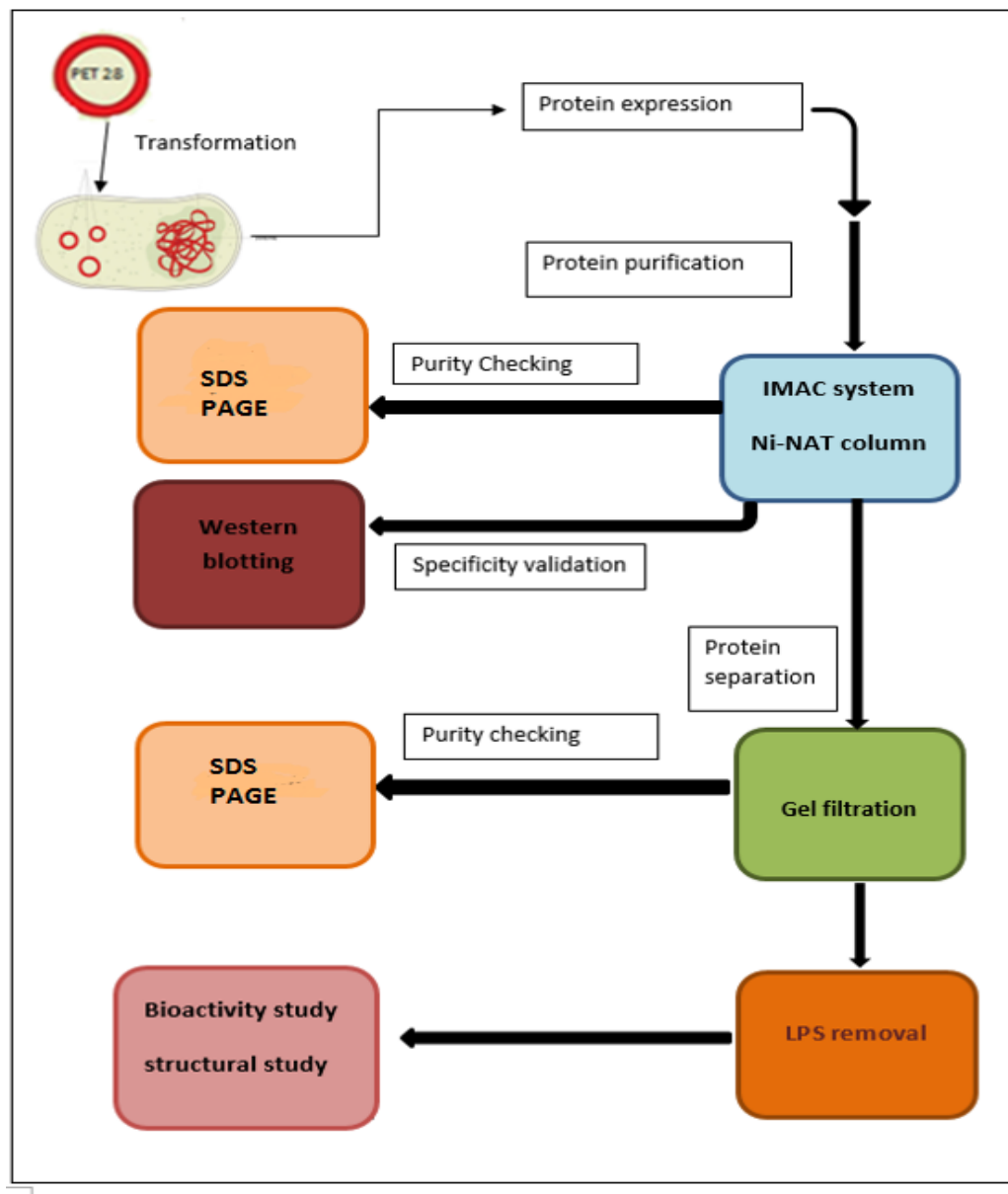


Figure 3.2 Strategy were used to produce human IL-37a and IL-37b recombinant proteins

The expression vector of each IL-37 isoform was transformed into *E. coli* BL21 (DE3) pLysS. Protein expression is induced by the addition of IPTG. The recombinant His-tag proteins are purified initially by Ni-NTA chromatography and then gel filtration. LPS is removed by Endotrap column. The purity, and specificity of the protein are confirmed by SDS-PAGE gel, western blotting, and mass spectroscopy respectively.

3.2.2.1 Plasmid construction for the expression of IL-37b in *E. coli*.

The plasmid encoding full length human recombinant IL-37b protein was generated by Dr Jiangning Liu in Dr Xu's lab (unpublished results). The plasmid pET-28/IL-37b contains human IL-37b gene fused with a DNA sequence that encodes 6histidine amino acids under T7 promotor at the C-terminal. Restriction sites includes the Xho I and Nde I for insertion of the gene of interest. Kanamycin sequence inserted as a selection marker (Fig 3.3).

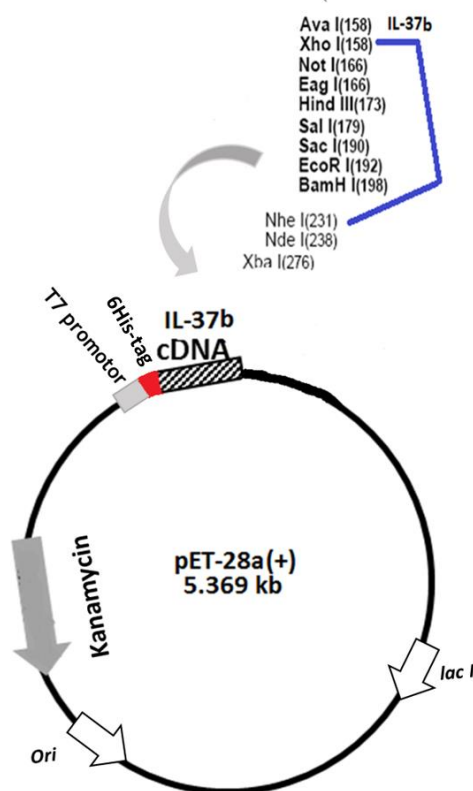


Figure 3.3 Schematic representation of pET-28a/IL-37b vector for IL-37b expression.

PET-28 a (+) construct was used to express recombinant IL-37b as N-terminal His-Tag fusion protein under T7 promoter. The vector contains the following elements or encoding sequences: replication origin (Ori); kanamycin resistant gene coding sequence; T7 promoter region; His-tag coding sequence; Lac I coding sequence; IL-37b cDNA was inserted into the plasmid between Nde I and Xho I sites.

3.2.2.2 Test expression of IL-37b

IL-37b was successfully produced in *E. coli*. Briefly, *E. coli* BL21 (DE3) pLysS transformed with the pET-28a/IL-37b vector, and the confirmed clone cultured overnight in a medium supplemented with kanamycin. To optimise expression recombinant IL-37b expression in bacteria cultured in log-growth phase induced with IPTG for vary time (from 1-7) hours. The levels of expression determined by SDS-PAGE as indicated in (Fig 3.4) by comparison to the non-induced bacterial culture. Accordingly, the best expression induction time for IL-37b was between (4-5) hours, and when the incubation time exceeded 5 hours; the yield of the product decreased significantly.

Recombinant protein expressed in *E. coli* can be soluble or insoluble (Sorensen and Mortensen, 2005). Recombinant cytokines expressed in *E. coli* are insoluble in inclusion bodies and this may make purification more complex. In order to purify the protein, the inclusion body has to be isolated and the protein solubilised by denaturation. Then after purification, the denatured proteins have to be renatured by removal of the denaturing agents (Sørensen and MortensenMany, 2005). Therefore, I further tested the expressed IL-37a and IL-37b proteins expressed in our *E. coli* system to determine if the expressed protein was produced in a soluble form. Briefly, the IL-37 expressing strains cultured and IPTG induced for 5 hours as above. The cell pellet was re-suspended in lysis buffer supplemented with lysozyme and protease inhibitors and sonicated until the solution was no longer viscous. The cell lysate centrifuged at 5000 xg for 30 min at +4 °C to separate the soluble and insoluble cell fraction. The IL-37a and IL-37b levels in the two fractions, respectively, determined by SDS-PAGE. While both fractions contained the 21 and 24 kDa bands, respectively, the majority of IL-37 proteins were found in the soluble fraction (data not shown). Thus, most IL-37a and IL-37b proteins expressed in this system are soluble.

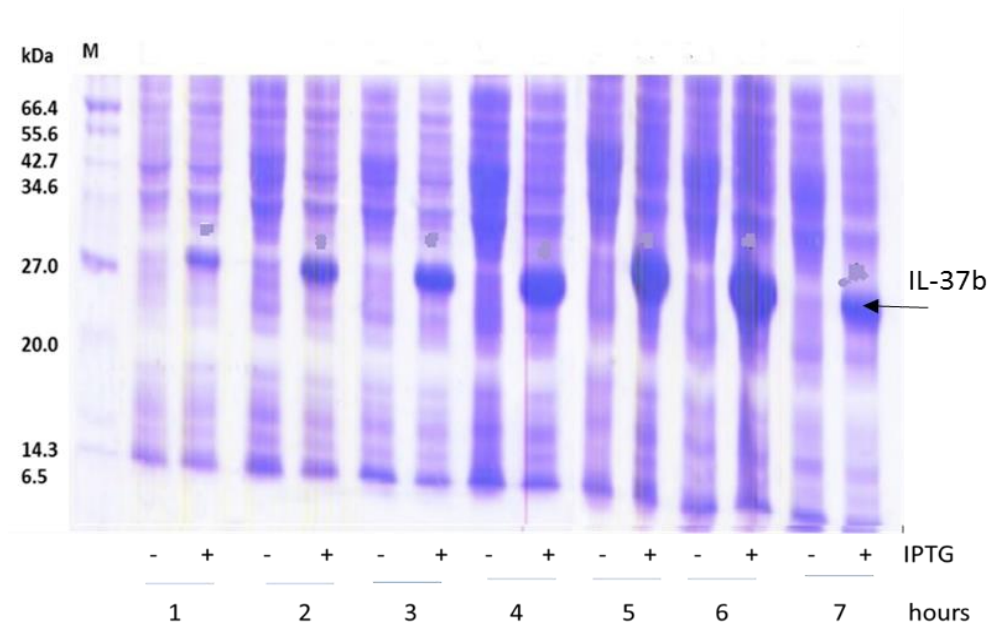


Figure 3.4 Test expression of recombinant IL-37b in *E. coli*.

The IL-37b expression induced in bacteria by 1 mM IPTG at 37°C for different times. The total bacteria were lysed, the protein separated by SDS-PAGE and the gel stained with Coomassie blue. Lane M indicates the markerbands in kilo Dalton, lanes numbered with (1, 2, 3, 4, 5, 6, 7) indicate the bacterial expression of IL-37b with or without IPTG; the arrow refers to rIL-37b band expression which is expected to be about 25 kDa. The result is representative of three independent experiments.

3.2.2.3 Purification of IL-37b by Ni-NTA chromatography

Detailed methods for the purification can be found in Chapter 2. Briefly, the bacteria expressing IL-37a were re-suspended in binding buffer supplemented with lysozyme and protease inhibitor. Bacteria were lysed by sonication and the cell debris removed by centrifugation. The supernatant containing the His-tagged IL-37b was loaded to the Ni-NTA column equilibrated previously with binding buffer for line A and elution buffer (binding buffer with 500 mM imidazole) for line B. The unbound proteins (without His-tag) removed using binding buffer for 35 minutes (Fig 3.5a). The bound His-tagged-IL-37 proteins eluted with a sharp peak using elution buffer containing 500 mM Imidazole for 25 minutes (Fig 3.5a). The IL-37b expression and purity checked by SDS-PAGE (Fig 3.5b). The bands at 25 kDa seen clearly in the fraction number 15-20 (Fig 3.5b). The protein markedly enriched in the eluted fractions compared to the loading and unbound samples. Thus, the results demonstrated that the His-tagged IL-37 proteins can be effectively produced in *E. coli* and successfully purified by Nickel Nitrilo-triacetic Acid (Ni-NTA) chromatography.

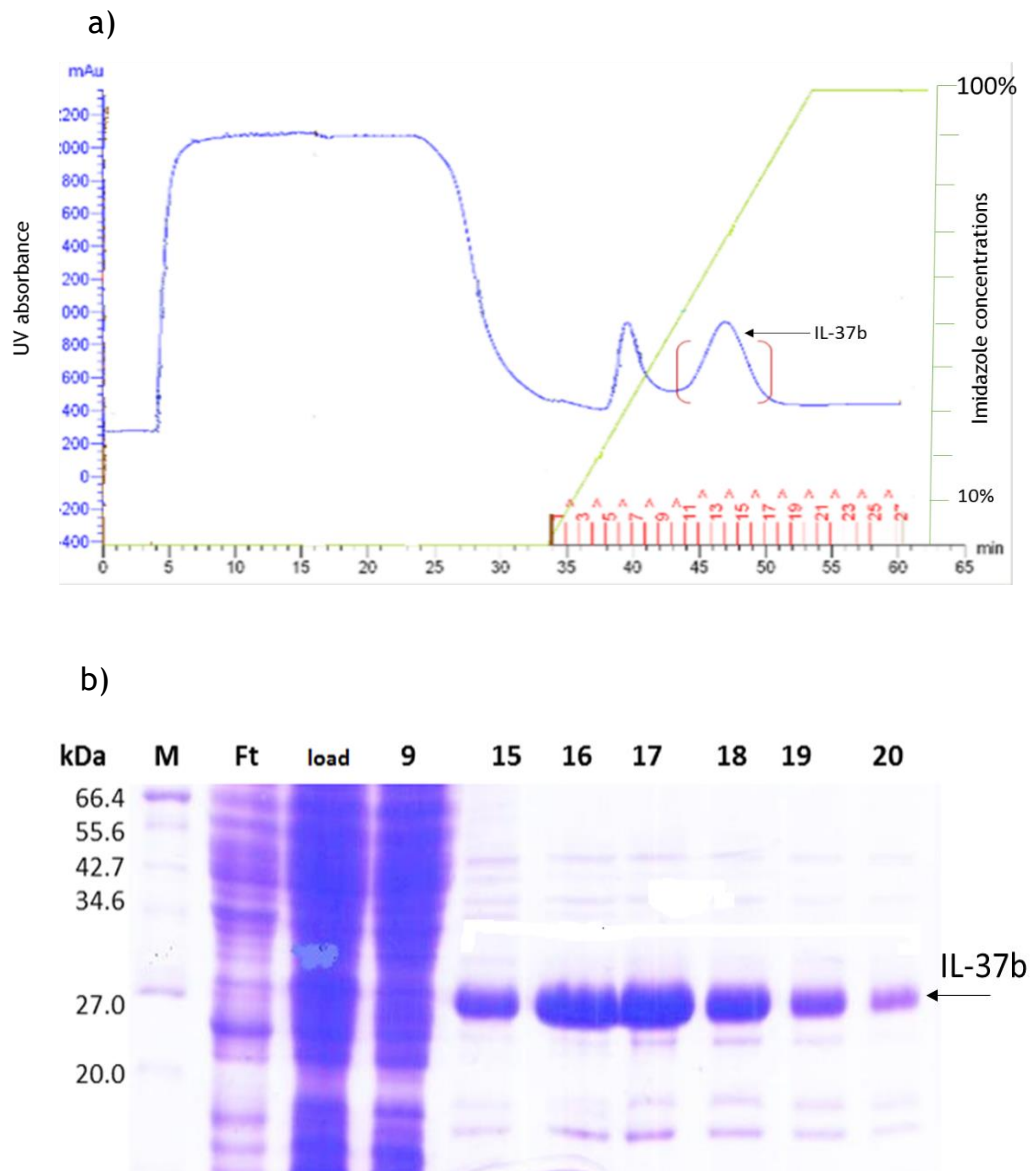


Figure 3.5 Nickel affinity chromatography for IL-37b purification.

6His-IL-37b was purified with His-Trap column (5ml) using the AKTA system. (a) 35 ml of soluble bacterial containing 6His-tagged-IL-37b extract loaded to the Ni-column at a flow rate of 1ml/minute and the UV absorption monitored at 280 nm; the IL-37b was eluted from the column using imidazole, as the peak with red brackets shows rIL-37b. (b) is SDS-PAGE gel. Fractions numbered (15 to 20) were collected, and re-suspended in SDS-PAGE loading buffer, heated at 95 C° for 10 minutes; separated on 15% SDS-PAGE. The gel stained with Coomassie Brilliant Blue R-250 solution, and de stained with de stain buffer. Lane M is the protein marker; Line Ft is the flow throw; load represents the bacterial extract containing rIL-37b; Lanes numbered (15-20) elution fractions of rIL-37b eluted by imidazole.

3.2.2.4 Confirmation of the His-tagged IL-37b by western blot

The identity of purified IL-37b was confirmed by western blot. As no commercial anti-IL-37 antibody was available for western blot, a well-characterised anti-6His-tag monoclonal antibody was employed for the purpose. The different fractions of IL-37b eluted by Nickel affinity chromatography were separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The proteins were then labelled with conjugated anti-6His-tag monoclonal antibody and detected by western blotting. As shown in (Fig 3.6), a single band with molecular weight of His-tagged IL-37b clearly detected in the samples, indicating that the purified protein is His-tagged IL-37b.

3.2.2.5 Purification of IL-37b proteins by gel filtration

To further purify IL-37b collected from IMAC affinity chromatography the protein was dialysed in (50 mM Tris and 200 mM NaCl; pH 7.5) and loaded on an Superdex-75 SEC column equilibrated in the same buffer and separated with flow rate 1.5 ml/minute (Fig 3.7a). The fractions (A10-15, B15, B14, and B4) were collected and analysed by SDS-PAGE (Fig 3.7b).

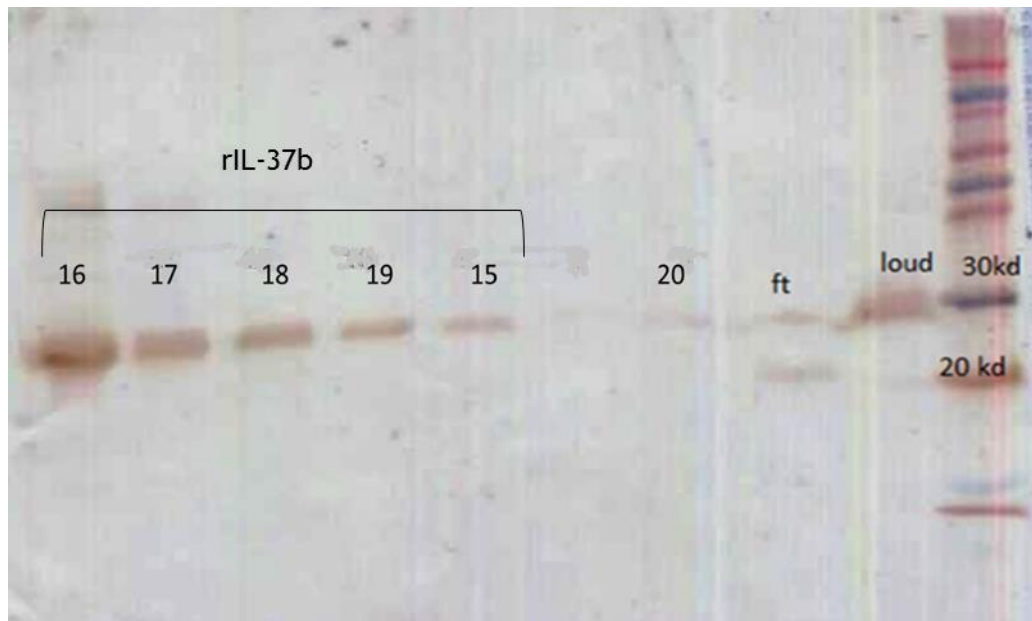
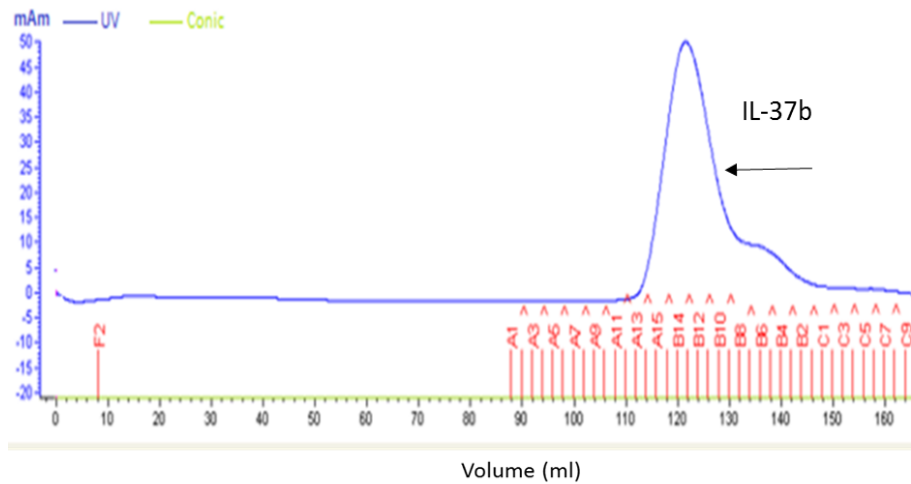


Figure 3.6 Confirmation of the His-tagged IL-37b by western blotting

The protein fractions from Ni-NTA affinity chromatography run by SDS-PAGE, transferred onto nitrocellulose membrane, and detected using conjugated anti 6His-tag antibody. The first line (M) indicates the molecular weight marker. Lane (load) is the loading sample before the Nickeled column; lane (Fth) is the unbound protein flow through the Nickel column. Lanes (15-20) are different fractions of 6His-tagged-IL-37b eluted from Ni-NTA column. The fractions numbers are the same as in the (Fig 3.5b). The experiment repeated three times.

a)



b)

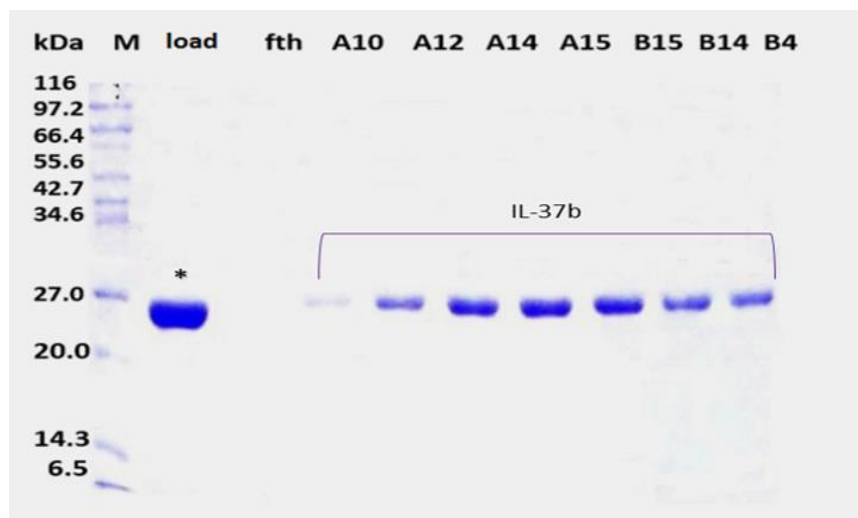


Figure 3.7 Purification of IL-37b by Gel filtration.

The Ni-NTA purified IL-37b samples are further purified by gel filtration. a) Dialysed IL-37b was loaded onto a Superdex-75 column; the protein separated in the column with a flow rate of 1.5 ml/minute. b) Fractions collected to test purity using SDS-PAGE; the collected fractions from gel filtration assessed by 15% SDS-PAGE and Coomassie blue staining. Lane (M): is the protein marker; Lane load: is the loaded sample and the band with the star represents rIL-37b; Lane

ft: washed away proteins. Lanes (A10, A12, A14, A15, B15, B14, and B4) are the protein fractions collected from the column (as in fig 3.7a). The experiment repeated three times.

3.2.2.6 IL-37b Circular Dichroism (CD)

Circular dichroism (CD) is a form of light absorption spectroscopy that measures the difference in absorbance of left handed and right handed circularly polarized light. This technique depends on the interaction between polarized light and an optically active matter (Greenfield, 2006).

First, plane polarised light is composed of two circularly polarised components that have equal magnitude, one of them is left handed light and the other is right handed light. Second, an optically active matter or chiral chromophore is an atom or molecule, which rotates polarised light in either a right hand (Dextro) or a left hand (Levo) direction. A chromophore to be chiral for these reasons:

- 1- Intrinsically chiral because of its structure is for instance an atom bound to four different groups or disulphide bound, because of the dihedradal angles.
- 2- The chromophore bound to a chiral centre.
- 3- The chromophore is in an asymmetric environment.

A chiral centre absorbs left and right-handed circularly polarized light differently, a phenomenon that exploited in CD spectroscopy for rapid determination of stability, structure of proteins, nucleic acids, and lipids. In proteins, the presence of peptide bond (absorption below 240 nm) make them optically active in addition to disulphide bonds (absorption around 260 nm). Different types of protein secondary structure give rise to CD in the far UV (180-260) nm. Structural information can be obtained at atomic level of resolution using nuclear magnetic resonance techniques (NMR) or X-ray crystallography, however; NMR needs high concentration of proteins (0.5 mM), high temperature, acidic pH, long time, and also it is limited to small proteins < 40 kDa. X-ray crystallography needs time for crystals to grow. In addition, it cannot be applied to membrane proteins, whereas CD takes 30 minutes (Kelly et al, 2005; Micsonai et al, 2015). There are some factors, which should be taken in account for

structural studies. Preparation of fused protein is of high importance since addition of tags can be problematic, some molecules affect the absorbance signal for instance glutathione -S-transferase (GST) or maltose binding protein (MBP) because they are big moieties of 26 kDa and 40 kDa respectively. The presence of nucleic acids, and protein aggregates in the CD samples also distort the magnitude of the signal. Reliable estimation of protein secondary structure by CD depends on accurate concentration and instrument calibration (Kelly et al, 2005). CD at different temperature is also an informative tool for protein stability.

Far-UV circular dichroism (CD) used to analyse protein secondary structure and the use of CD at different temperatures is an informative tool to analyse protein stability. The technique was applied to recombinant IL-37b after purification (Fig 3.8a and b). The secondary structure prediction from circular dichroism indicated that the IL-37b does not contain significant stretches of α -helix and the protein showed aggregation after 40 °C.

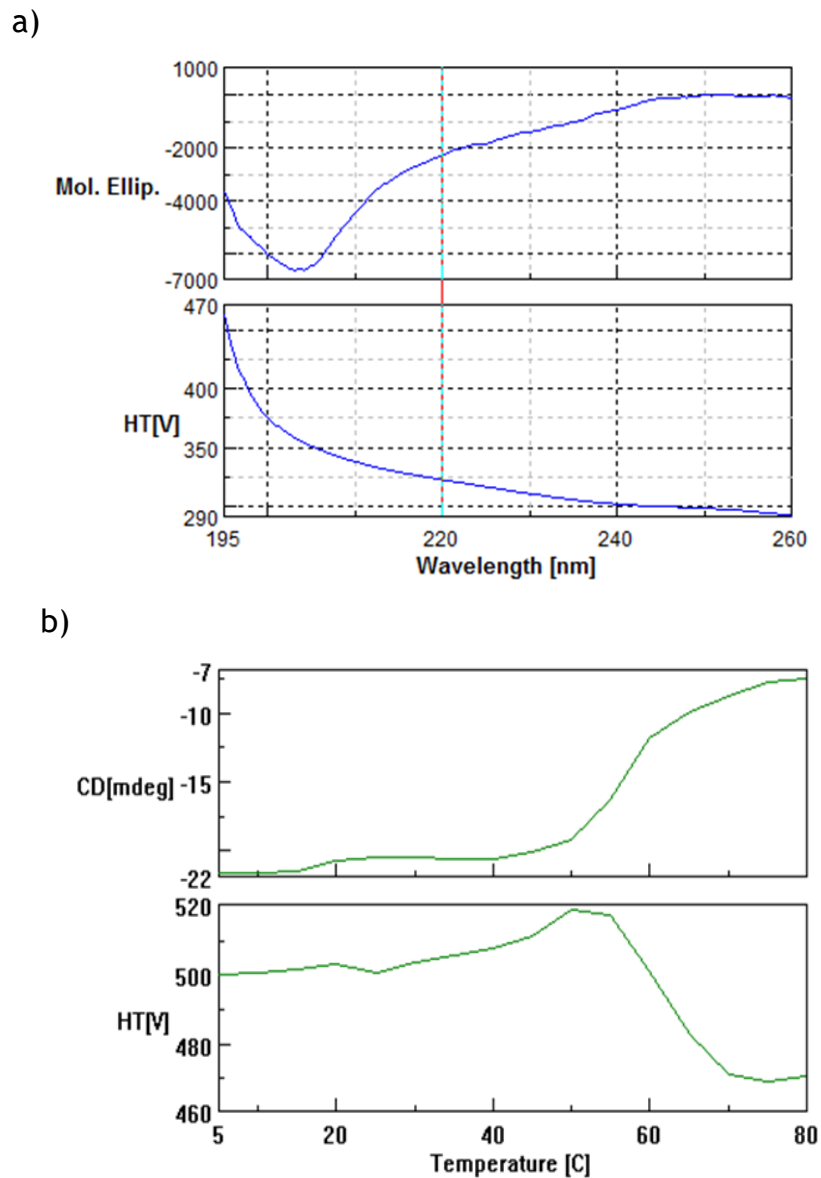


Figure 3.8 Far UV CD spectrum of IL-37b

a) Spectra were recorded on a Jasco J-710 spectropolarimeter in a 0.02 cm path length cell at 20 degrees C. UV A280 0.3678: Protein concentration 0.437 mg/ml. Mean residue weight concentration = $0.437/\text{mrw} (111.88) = 3.90597\text{e-}3\text{M}$. b) Thermal melting of IL-37b. Spectra were recorded at 5 °C intervals from 5 to 80 degrees C. Data were plotted using the ellipticity value at 206nm. Note the increase in HT (High Tension) values above 40 degrees C. This increase is indicative of light scattering as the protein begins to aggregate. The decrease in HT at temperatures above 60 °C reflects the movement of the scattering particles out of the light path.

3.2.3 Production of recombinant IL-37a

3.2.3.1 Plasmid construction for the expression of recombinant IL-37a in *E. coli*.

The plasmid encoding FL human recombinant IL-37a in *E.coli* was generated by Dr Jiangning Liu in Dr Xu's lab (unpublished results). The plasmid pET-21a/IL-37a (Fig 3.9) encodes human IL-37a fused with a sequence encoding 6 histidine amino acids at the 5'-end, inserted via the restriction enzymes Xho I and NdeI cutting sites at the 3' and 5' end, respectively. The plasmid contains an ampicillin resistant gene for selection and preventing of bacterial contamination.

3.2.3.2 Test expression of IL-37a in *E. coli*

While the recombinant IL-37b expression successfully produced in *E. coli*, whether recombinant IL-37a produced in *E. coli* is unknown. The plasmid pET21 a/IL-37a construct (Fig 3.9). The IL-37b construct used as a positive control; and the same steps to the BL21 (DE3) pLysS strain transformed with pET21/IL-37a 3-3-2 Test expression of IL-37b. The levels of expression determined by SDS-page electrophoresis as indicated in (Fig 3.10), compared to the non-induced bacterial controls. The size of the protein was approximately 21 kDa, which is compatible with the theoretical molecular weight of human IL-37a. The kinetics of study indicates that the IL-37a expression was induced 1 hour after the addition of IPTG; and the optimal induction time was between 4-5 hours; which is not different from IL-37b.

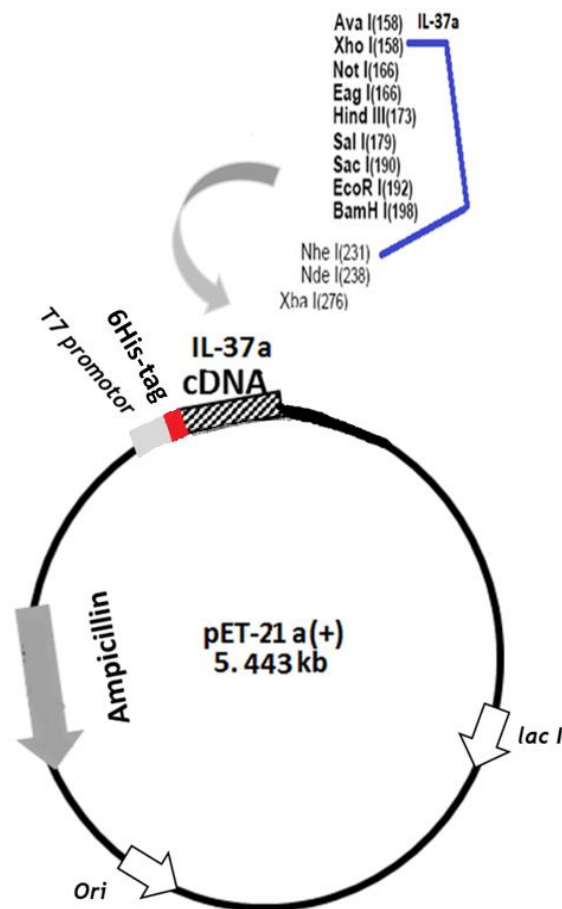


Figure 3.9 Schematic representation of pET- 21a/ IL-37a construct.

The pET- 21 a (+) vector used to express recombinant IL-37a as N-terminal His-tag fusion protein under T7 promoter. The vector contains the following key elements and encoding sequences: replication origin(Ori); ampicillin resistant gene coding sequence; T7 promoter region; IL-37a gene; 6His-tag coding sequence; LacI coding sequence; IL-37a cDNA was inserted into the plasmid between Nde I and Xho I sites.

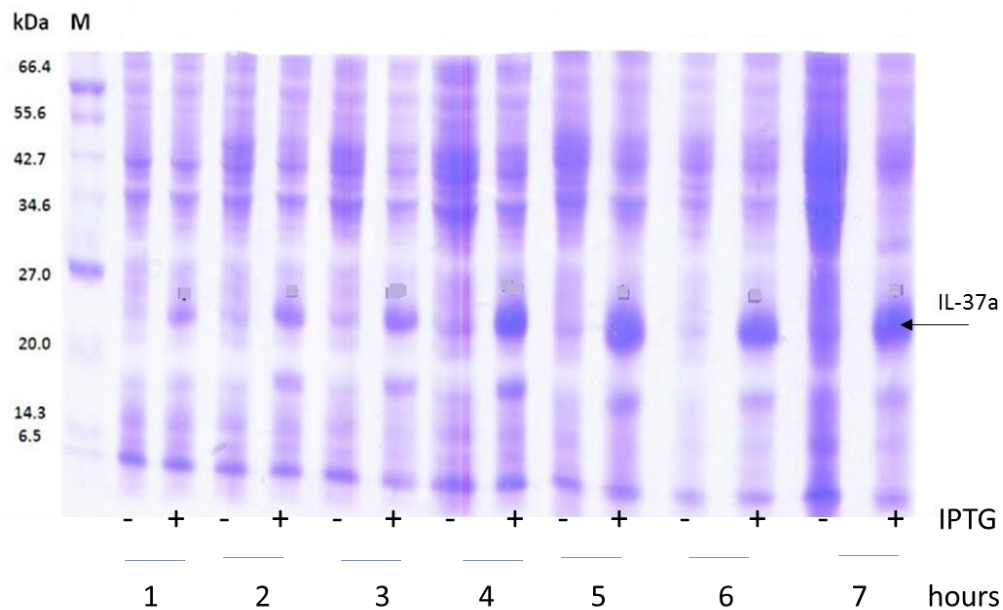


Figure 3.10 Test expression of recombinant IL-37a in E- coli

The IL-37a expression induced in bacteria by 1 mM IPTG at 37 °C for different periods. The total bacteria lysed, and soluble proteins separated by SDS-PAGE electrophoresis and the gel stained by coomassie brilliant blue. Lane M indicates the marker used in kilo Dalton (kDa) illustrated at the left; lanes numbered with (1, 2, 3, 4, 5, 6, 7) indicate the bacterial expression of rIL-37a with or without IPTG; the arrow refers to rIL-37a band expression which is expected to be about 22 kDa. The experiment repeated five times.

3.2.3.3 Purification of IL-37a by Ni-NTA chromatography

The bacterial pellet expressing IL-37a, re-suspended in binding buffer supplemented with lysozyme and protease and the supernatant containing the

His tag-fusion IL-37a protein loaded to the Ni-NTA column (Fig 3.11a). The same IL-37b His-tag procedure used for the purification of IL-37a by Ni-NTA chromatography. However, the time used for the purification of IL-37b was much shorter than that of IL-37a. The washing step in IL-37a purification completed within 60 to 120 minutes; and the bound His-tagged-IL-37a protein eluted within 120-190 minutes. Based on the protein optical density (OD) and experiences, the fractions of the protein elution from 17-26 were collected.

3.2.3.4 Confirmation and purity of the His-tagged IL-37a protein by SDS-PAGE

I next checked if the eluted protein fractions (17-26) contain IL-37a protein, and what is the purity using SDS-PAGE (Fig 3.11b). The result showed that the protein with the molecular weight about 21 kDa in the bacterial extract could bind to Ni-NTA and eluted by imidazole, indicating that it is the 6His-tagged IL-37a protein. While this protein band seen in all the collected fractions (number 17, 19, 22, 23, 24, 25, and 26), the majority of the proteins existed in the fractions 17, 19 and 22. Compared to the loading samples, the Ni-NTA chromatography effectively enriched the recombinant protein from the bacterial lysis. However, this protein seen in the unbound fractions (line 3) or the flow throw from the column, this suggests that the loading quantity and condition were further optimised.

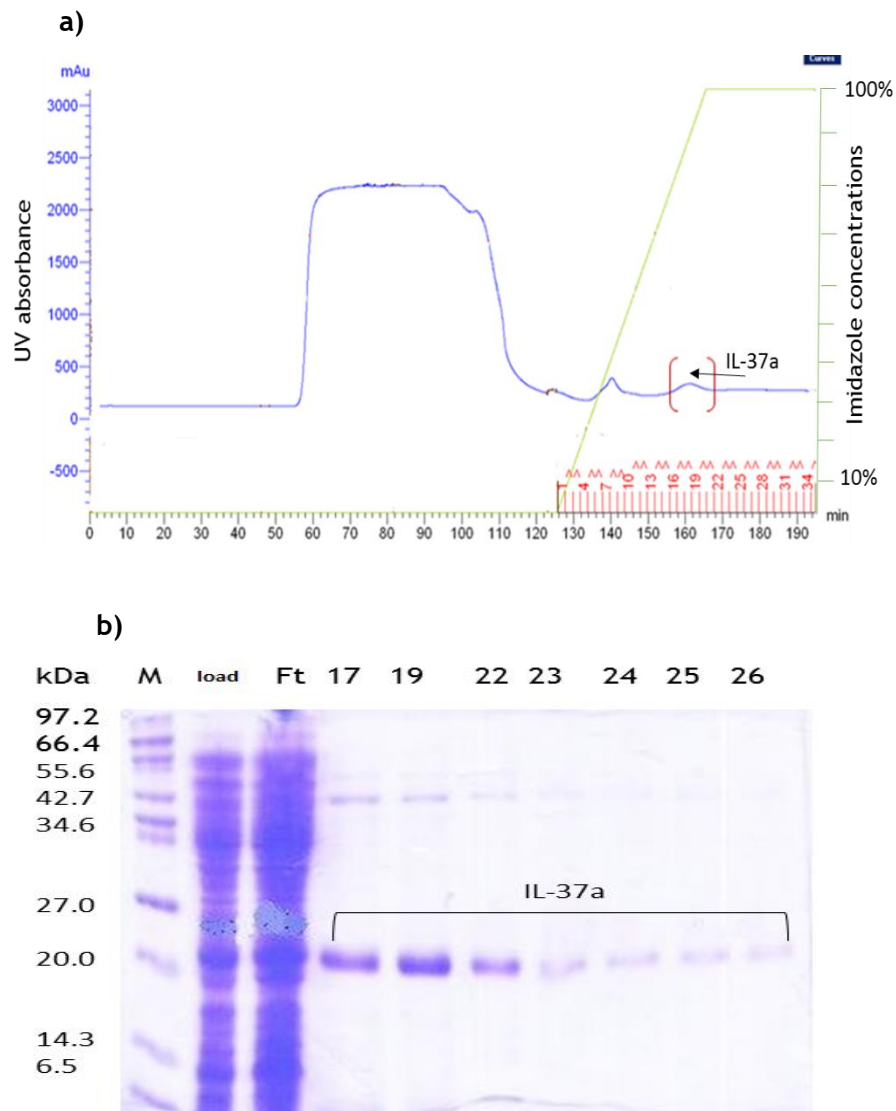


Figure 3.11 Nickel affinity chromatography for IL-37a purification.

a) IL-37a- 6His-tagged protein purified with His-Trap (5ml volume column) using the AKTA system. 35 ml of soluble bacterial containing IL-37a- 6His-tag extract loaded to the Ni- column, with 1ml/min flow rate; and the absorption monitored at 280 nm; the peak with the red brackets is IL-37a protein. b) SDS-PAGE gel. Fractions numbered (17 -26) collected and re-suspended in SDS-PAGE loading buffer, heated at 95 C° for 10 minutes; then separated on 15% of SDS-PAGE gel. The gel stained with 0.1% Coomassie Brilliant Blue R-250 solution and de stained with de stain buffer. The first line (M) is the marker with the molecular weight on the left, lane (load) is the protein extract loaded, and lane (ft) is the unbound flow throw proteins out from the column, respectively. Lane numbered (17, 19, 22, 23, 24, 25, and 26) correspond to 6His-tagged- IL-37a.

3.2.3.5 Confirmation of the His-tagged IL-37a proteins by western blot

Since the protein size in (Fig 3-12) can only suggest, but not ensure that the purified protein is IL-37a-His protein, I next confirmed this by western blot. As the same was done for IL-37b His-tag in section 3.3.5, the different fractions of His-tag IL-37a protein eluted by Nickelled affinity chromatography separated by SDS-PAGE and transferred onto nitrocellulose membrane. The protein was labelled with conjugated anti-6His-tag monoclonal antibody, and detected by western blotting. As shown in Fig 3.12, a single band with molecular weight of His-tagged IL-37a clearly detected in the samples, further; suggesting that the proteins is His-tagged IL-37.



Figure 3.13 Confirm the His-tagged IL-37a by western blotting.

The protein fractions from Nickel affinity chromatography run by SDS-PAGE, transferred onto nitrocellulose membrane, and the His-tag IL-37a was detected using conjugated anti 6His-tag antibody. The first line (M) indicates the marker with the molecular weight (kDa); the second lane (rIL-37a) is recombinant IL-37a used as positive control, lane (load) is the loading sample on the Nickeled column before purification; lane (Fth) is the unbound proteins flow throw through the Nickel column. Lanes (25, 24, 23, 22, 19, and 17) are different fractions of rIL-37a eluted from the Nickeled column. The fractions numbers are the same as in the (Fig 3.11b). The experiment repeated three times.

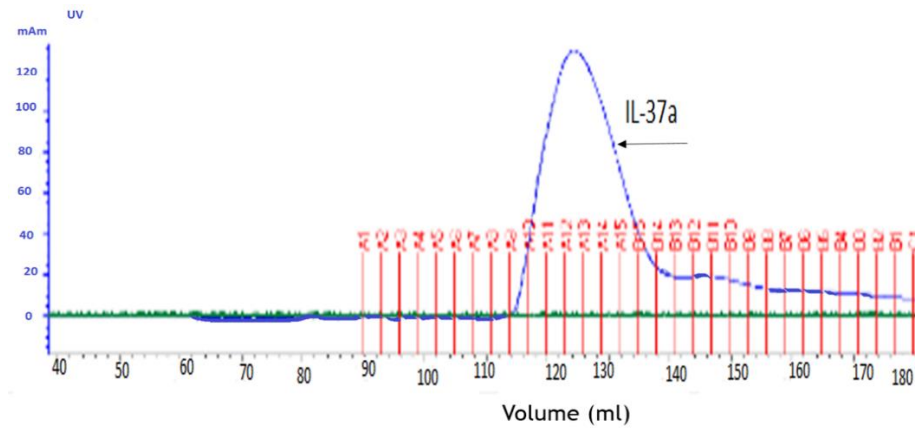
3.2.3.6 Purification of IL-37a proteins by gel filtration

As seen in (Fig 3.11), while the Nickel affinity chromatography was effective in the enrichment of recombinant IL-37a protein, many contaminating proteins were still present in the protein preparations. Next, the contaminants of proteins were further removed by size exclusion chromatography (SEC). The separation with SEC performed under native conditions to preserve the biological activity of the protein.

After the successful purification of IL-37b, however, the nickel purified IL-37a proteins precipitated during the dialysis, which were seen by naked eyes. The precipitation of the protein led to the loss of considerable amount of IL-37a (up to 50%). Several dialysis conditions were developed to overcome this problem (Section 3.5.1).

IL-37a was further purified by SEC; using the same conditions as IL-37b described above. As shown in the (Fig 3.13a), a clear protein peak was detected after the sample loaded; and the protein fractions correlated to the protein peak collected for the purity checking using SDS-PAGE as before.

a)



b)

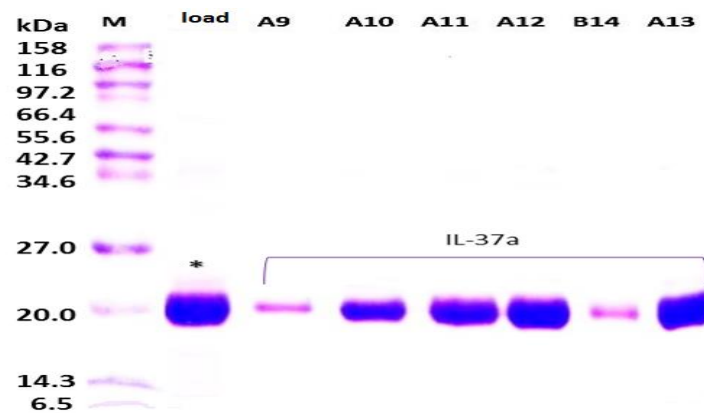


Figure 3.14 Purification of rIL-37a by Gel filtration

The Nickeled purified IL-37a samples were dialysed in 50mM tris and 200 mM NaCl, and loaded onto gel filtration column. a) The proteins were separated using S-75 sephadex column with 1.5 ml/minute flow rate; and the protein peak fractions collected for SDS-PAGE gel. b) Represents collected fractions from gel filtration assessed by 15% SDS-PAGE; Line M: the protein marker. Line load: loaded sample. Lanes (A9, A10, A11, A12, A13, and B14) are the protein fractions collected from the column.

3.2.3.7 Identity confirmation of purified IL-37a by mass spectrometry

Mass spectrometry (MS) is a powerful technique with different applications in chemistry, biology and clinical medicine. It determines the molecular weight of compounds by separating molecule ions based on their mass and charge. Recently MS has become one of the most important methods for proteins studies in term of identification and sequencing.

The mass spectrometry strategy composed of three main steps:

- 1- Analyte ionization: ionization converts the molecule to ions. Electrospray ionization (ESI), matrix-assisted laser desorption and ionization (MALDI) are the most commonly used methods in proteomics.
- 2- Analysis of mass: molecules separated according to their mass/ charge ratio (m/z), in proteomics the mass analyser can be an ion trap, an fourier transform ion cyclotron resonance (FTICR), hybrid of the aforementioned a or a quadrupole analysers.
- 3- Detection of the output: signal is recorded at the detector, and results can be m/z vs intensity plot, or a mass spectrum. The detector is normally an electron multiplier. The mass analyser and detector are always within the high-vacuum region (Zhu et al, 2010; Ilavenil et al, 2016).

Whereas the results of SDS-PAGE and western blot using anti-6His-tag antibody in (Fig 3-11b and 3-12); indicated that the purified proteins were IL-37a, this result is still unable to define the protein identity. Next, we decided to determine the protein identity by MS. IL-37a band with molecular weight of IL-37a in (Fig 3.11) was cut out from the SDS gel. The protein in the gel pieces was enzymatically digested using trypsin into peptide fragments. The protein sequence of the protein fragments was determined by mass spectrometric analysis (Sutton et al, 1995). MS demonstrated that the resulted peptide sequences covered 84.375% of the published human IL-37a sequence (Accession number (Q9NZH6-2) with 100% homology (Table 3.3 and data not shown). Thus, the purified protein is human IL-37a. Amino acids sequences detected by MS, also confirmed that the purified protein is human IL-37a (Table 3.3).

Table 3.3 Mass Spectroscopy confirms the protein sequence of purified human IL-37a.

Accession Q9NZH6- 2	score/ 6616.46	Coverage/84.38%
A2	Sequence	# PSMs
High	VLVLDSGNLIAVPDK	44
High	NYIRPEIFFALASSLSASA EK	44
High	VLVLDSGNLIAVPDKNYIR	2
High	AQVGSWNLESAAHPGWFIcTScNcNEPVGVTDFENR	7
High	AQVGSWNmLESAAHPGWFIcTScNcNEPVGVTDFK	3
High	NYIRPEIFFALASSLSASA EKGPILLGVSK	7
High	LAAQKESAR	1
High	VLVLDSGNLIAVPDKNYIRPEIFFALASSLSASA EK	12
High	HIEFSFQPVcK	23
High	GPILLGVSK	14
High	FSIHDQDHKVLVLDSGNLIAVPDK	1
High	GPILLGVSKGEFcLYcDK	2
High	AQVGSWNMLESAAHPGWFIcTScNcNEPVGVTDFK	2
High	AQVGSWNmLESAAHPGWFIcTScNcNEPVGVTDFK	3
High	GEFcLYcDKDK	8
High	GEFcLYcDK	2
High	HIEFSFQPVcKAE mSPSEVSD	1
High	KHIEFSFQPVcK	2
High	GEFcLYcDKDKGQSHPSLQLK	5
High	KFSIHDQDHK	1
High	AE mSPSEVSD	1
High	AQVGSWNMLESAAHPGWFIcTScNcNEPVGVTDFENR	1
High	KFSIHDQDHKVLVLDSGNLIAVPDK	1
High	AQVGSWNmLESAAHPGWFIcTScNcNEPVGVTDFENR	3
High	DKGQSHPSLQLK	1
Medium	GPILLGVSKGEFcLYcDKDK	1
Medium	RPFIFYR	5
Medium	GQSHPSLQLK	2
Medium	DKGQSHPSLQLKK	1
High	AEMSPSEVSD	2
High	AE mSPSEVSD	2
Medium	AQVGSWNMLESAAHPGWFIcTScNcNEPVGVTDFENRK	1
Medium	GQSHPSLQLKK	1
Medium	VKNLNPK	1

The protein sequences identified by mass spectroscopy matched with the known protein sequence of human IL-37a (Q9NZH6-2).

Mass spectroscopy analysis confirmed that the protein was intact, and covers 84.38% of human IL-37a sequence. The sequence below shows human IL-37a amino acids sequences and the highlighted sequences with red indicate similarity between the sequences in Table 3.3 with human IL-37a (Q9NZH6-2).

MSGCDRRETETKGKNSFKKRLRGPKVKNLNPKKFSIHDQDHKVLVLD SGNLI AVPDKNYIRPEIF
 FALASSLSSASAEKGSPILLGVSKGEFCLYCDKDKGQSHPSLQLKKEKLMKLAAQKESARRPFIFY
 RAQVGSWNMLESAAHPGWFICTSCNCNEPVGVTDKFENRKHIEFSFQPVKAEMSPSEV SD.

3.2.4 Optimisation of IL-37a and IL-37b protein purification

3.2.4.1 Dialysis conditions

In initial experiments, Ni-NTA purified IL-37a precipitated extensively during dialysis, led to loss of protein; to overcome this drawback, I tried several dialysis conditions in an attempt to improve solubility, and to prevent protein precipitation (Table 3.4). The protein showed great stability in dialysis buffers contains (250 mM NaCl Tris 50 mM) with pH= 7.5, and less precipitation noticed.

Table 3.4 Dialysis conditions used to improve IL-37a solubility

Conditions used for dialysis	concentration	
	before dialysis	After dialysis
250 mM NaCl+ Tris 50 mM (pH=7.5)	1.191	1.065
500 mM NaCl+ Tris 50mM (pH=7.5)	1.191	1.001
(1mM DTT) + (250 mM NaCl) + Tris 50mM (pH=7.5)	1.191	0.978
(1mM DTT) + (500 mM NaCl) (pH=7.5)	1.191	0.905
(Glycerol 5%) + (250 mM NaCl) (pH=7.5)	1.191	0.94
(Glycerol 5%) + (500 mM NaCl) (pH=7.5)	1.191	0.901
PBS (137 mM NaCl; 2.7 mM KCl; 8 mM Na ₂ HPO ₄ ; and 2 mM KH ₂ PO ₄ , pH=7.5)	1.191	0.552
Tris buffer (pH=7.5)	1.191	0.993

3.2.4.2 Assess the efficiency and yield of recombinant IL-37 purification

The quantity and yield of the proteins in each of the purification steps of the production process was also determined and showed in the (Table 3.5) below. Nickel affinity chromatography was most the effective step that removed most of the contaminating proteins. The final yields of IL-37a and IL-37b were 1.2 mg and 2.4 mg, respectively.

Table 3.5 Recombinant IL-37a and IL-37b production yield after each purification step.

Cytokine	IL-37a (mg)	IL-37b (mg)
Yield of protein after Ni-column	10.3	14.6
Yield of protein after gel filtration	3.03	5.37
Yield of protein after LPS removal	1.2	2.4

The result is representative of 6 independent experiment.

3.2.4.3 Endotoxin removal

LPS, or (endotoxin), is an abundant surface component of gram-negative bacteria. It is also a powerful immune activator and can trigger and exacerbate many inflammatory and autoimmune disorders (Dullah and Ongkudon, 2016).

Bacterial LPS contamination in recombinant protein preparation is a potential problem in the application of the protein agent. Thus, the contaminated endotoxin was removed. Endotoxin in the purified IL-37a and IL-37b samples was removed with polymyxin B columns following the manufacturer's instruction. Levels of endotoxin in the IL-37 proteins preparations was measured before and after the LPS removal, using Limulus amoebocyte lysate (LAL) QCL-1000 pyrogen test kit (Chapter 2). Before LPS removal, the level of LPS in IL-37a and IL-37b were 0.83 EU/ μ g and 0.861 EU/ μ g of protein respectively, and after LPS removal, the level of LPS in both proteins were less than 0.01 EU/ μ g. This level of LPS in the cytokine preparations as acceptable for the bioactivity studies.

3.2.5 Crystallization of IL-37a and IL-37b protein

Protein structure prediction with experimental methods is determined by using X-ray crystallography (Gromiha M, 2004). Protein crystallization includes the creation of a supersaturated solution in conditions where do not perturb the proteins natural state. The production of super saturated conditions can be achieved through: 1) the addition of mild precipitating agent such as salts or polymers. 2) The use of various parameters; including pH, temperature; and ionic strength. Crystallization proceeds in two distinct inseparable steps; the first step is nucleation and the second step is growth. A variety of methods developed to affect and promote crystallization including dialysis, vapor diffusion, batch and liquid-liquid diffusion (Luft et al, 2014).

IL-37b isoform purified previously by Nickeled column and gel filtration in sections 3.3.3 and 3.3.5, dialysed in PBS buffer (Table 3.5); in which the protein remained stable and soluble. IL-37b protein was concentrated into 14 mg/ml using Sartorius Vivaspin (50 m tube from thermos Fisher Company); the protein kept on ice until processed to crystallization screen. Next, two different concentrations (14 mg/ml and 7.5 mg/ml diluted in PBS buffer) of IL-37b were used with different high throughput crystallization screens including JCSG-plus-HT-96, MIDAS-96; Morpheus- HT-96; and PACT premier-HT-96 (Molecular Dimensions Company). The screens were monitored every week for crystals formation by light microscopy. Most of the wells resulted in phase separation (Fig 3.14).

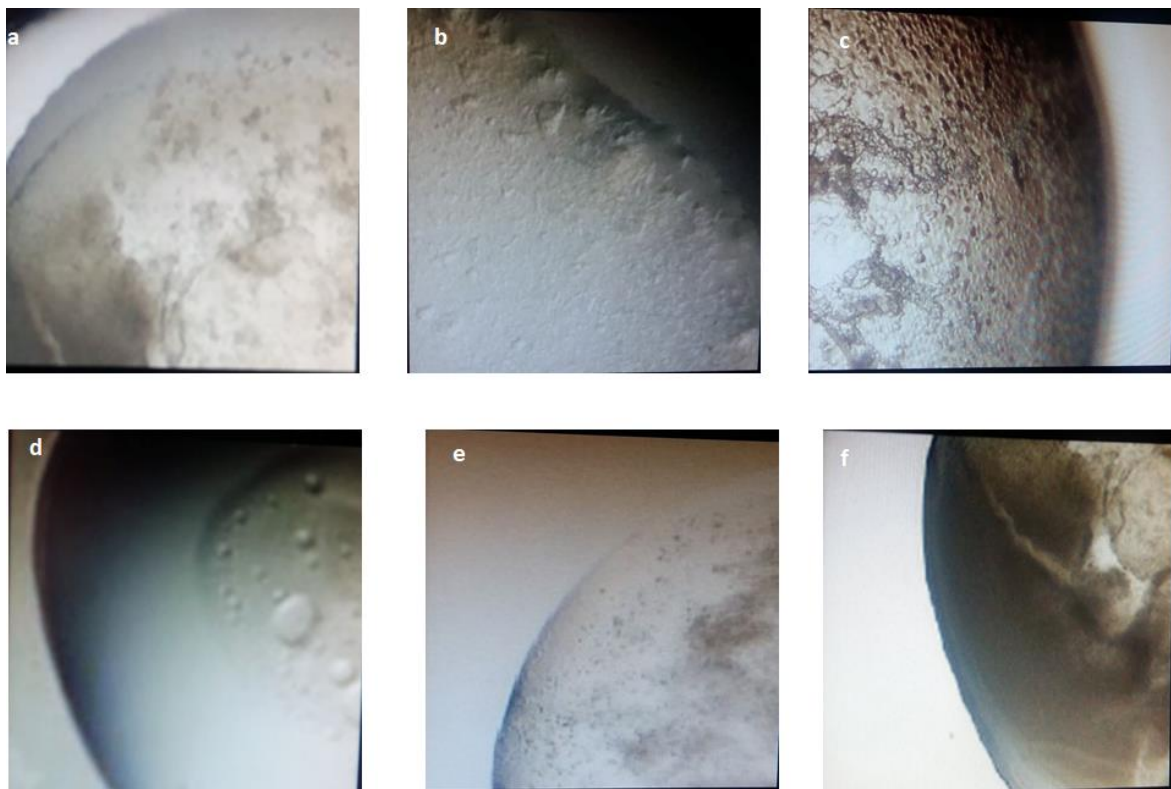


Figure 3.15 The results from IL-37b crystallization screens.

IL-37b was prepared for crystallization with two different concentrations (14 and 7.5 mg/ml), diluted in PBS buffer. a) granular precipitate(JCSG- plus-HT-96 screen with 14 mg/ml), b) granular precipitate (PACT premier-HT-96, with 14 mg/ml), c) granular precipitate (MIDAS-96, with 14 mg/ml), d) phase separation (Morpheus- HT-96, with 14 mg/ml), e) granular precipitate (JCSG- plus-HT-96 screen, with 7.5 mg/ml), f) phase separation (Morpheus- HT-96, with 7.5 mg/ml, with 7.5 mg/ml).

3.3 Discussion

Plasmids for the expression of IL-37a and IL-37b were successfully constructed and purification methods for the recombinant IL-37a and IL-37b were established. Both of these His-tagged proteins were successfully purified to a high level of purity with good yield. The choosing of BL21(DE3) pLysS as a host expression, resulting in good expression to give a final yield of purified protein of 10-13 mg/5L of culture.

The expression of the protein was not problematic, but IL-37a precipitation during dialysis was noticed. Several conditions were used to overcome IL-37a precipitation (Table 3.3) to optimize purification.

IL-37a is less soluble compared to IL-37b, and it was suggested that may be due to the isoelectric point (PI) of IL-37a which is 9.2 in comparison with IL-37b (PI 6.1). However, the buffer used was pH 7.5, which was at least one unit far from 9.2 to avoid protein precipitation. Therefore, I tried different salt concentrations with the same pH 7.5. Finally, by reducing the salt concentration, the protein exhibited stability in 250 mM NaCl with a very small amount of precipitation.

Western blotting was used to confirm the identity of the His-tagged proteins. The protein purity after Ni-column was relatively high and the remaining contaminants were removed by gel filtration. IL-37a after gel filtration had not shown clear dimerization, while dimerization of IL-37b was noticed. Fractions of IL-37b after gel filtration were tested with SDS-PAGE to check the purity and monomer fractions collected separately from the dimerised fractions.

IL-37b dimerization due to two main chains of hydrogen bonds formed between Tyr⁸⁵ and Arg⁸⁷ from each IL-37b monomer, and this interaction is protected by conserved ionic interactions between Lys⁸³ and Asp⁷³, in addition to extra hydrogen bonds formed between Ile⁸⁶ main-chain carbonyl group and Arg⁸⁷ side chain of each monomer. Tyr⁸⁵ mutation at the hydrophobic core is disrupted the dimer formation completely and results in a variant with a MW of ~18,000 kDa, suggesting that Tyr⁸⁵ is highly conserved and is a key role in the dimer stabilization; however, mutation of Asp⁷³ affect the ionic interactions gate of

the interface; thus, Asp⁷³ mutation moderately destabilizes IL-37b dimerization (Ellisdon et al, 2017).

Circular dichroism confirmed that the protein secondary structure of IL-37b seems to be unfolded at its native conditions, and the protein aggregated at temperatures above 40°C.

Mass spectrometry determination confirmed the IL-37a identity, and showed that IL-37a was intact and had not suffered from degradation during the purification process.

Crystallisation method was established, however, the protein was not crystallized before, I selected several screening conditions which were reported by Krumm et al (2015) to crystalize IL-18 cytokine, as IL-18 cytokine belong to the same family of IL-37b; but, IL-37b did not yield crystals, and most of the conditions gave phase separation (Krumm et al, 2015). IL-37 crystallization reported by Ellisdon et al (2017), while I was trying to find the suitable conditions for IL-37b crystallization. This group used hanging-drop vapor diffusion in (2.1 M ammonium sulfate, 0.1 M sodium acetate at pH 4.5), and the IL-37b crystals were grown at +20°C in flash cooled in liquid nitrogen (Ellisdon et al, 2017), while we were growing crystals at +17 °C in the incubator.

Chapter 4

Expression and function of IL-37a and IL-37b in immune cells

4 Expression and function of IL-37a and IL-37b in immune cells

4.1 Introduction

The optimal and preferential induction of IL-37 isoforms in immune cells is still poorly understood. There is one report, which shows that IL-37 isoform expressions in total human PBMCs are differently regulated by TLR stimulation within 8 hours (Rudloff et al, 2016). TLR1, 2, 3, 4, 5, 6, and 9 ligands upregulate IL-37b and c in these experimental condition and the most effective TLR ligand that induces IL-37 isoform expression is LPS (TLR4 ligand). TLR7/8 agonists had no effect on the induction of IL-37 expression. IL-37c expression is predominantly induced by PAM3 (TLR2 ligand), while IL-37a and e are not upregulated with these stimuli. However, the time effect beyond 8 hours has not been studied. Furthermore, this study also shows that monocytes comprise the main source of IL-37 (73%), followed by myeloid dendritic cells (mDCs) (12%), B cells (0.556%) and T cells (0.46%). Stimulation with LPS did not affect IL-37 expression in the cell populations (Rudloff et al, 2016). While this information is valuable, the relative expression of IL-37 isoforms in these immune cells is unknown. In addition, the details of qPCR primers for each of the IL-37 isoforms used in the study were not provided.

While the function of IL-37b has been well studied, the effect of other isoforms on immune response is still unknown. IL-37b are inflammatory signal an induced and feedback suppresses ongoing inflammatory response (Nold et al, 2009; Li et al, 2017; Zhan and Zeng, 2017). Whereas mice do not express IL-37, they express IL-1R8 and IL-18Ra; the IL-37 receptors can response to human IL-37 (Wang et al, 2018). This has made mouse a useful model system to study human IL-37 function in vitro and in vivo. Taking this advantage, human IL-37b transgenic mice have been developed and facilitated to study human diseases in vivo (McNamee et al, 2011; Davis et al, 2017; Liu et al, 2018)

Since IL-37 receptors are widely expressed on immune cells, IL-37b has profound effect on a wide range of innate and adaptive immune cells, including monocytes, macrophages, epithelial, NK, mast and T cells (Fehervari, 2014;

Zhao et al, 2014; Shuai et al, 2015; Wu et al, 2015; Wang et al, 2016; Zhang et al, 2017; Conti et al, 2018). However, its effect on mature B cells has not been studied, while it shown that IL-37 may prevent B cell progenitors to develop into B lymphopoiesis by reducing inflammation in bone marrow of aging mice (Henry et al, 2015).

B cells are the important lymphocytes in immunity. The main function of B cells is to produce antibody and mediate humoral immune responses (Amlot et al, 1985; Cerutti and Cols, 2013; Tsiantoulas et al, 2014). However, B cells can also serve as a professional APC to present antigen to T cells (Rivera et al, 2001; Rodríguez-Pinto, 2018). Furthermore, B cells after activation can also regulate other cell function by secreting a range of cytokines and chemokines (Fillatreau et al, 2002; Shen et al, 2014; Lino et al, 2015; Shen and Fillatreau, 2015; Vazquez et al, 2015).

The activation and differentiation of naive into effector B cells requires at least three signals: the BCR/antigen interaction, co-stimulation from T cells, in particular CD40/40L ligation, and cytokines from other cells and the B cell itself (Nonoyama et al, 1993; Parker, 1993; Wortis et al, 1995; Dorner and Radbruch, 2007). The CD40/CD40L ligation can induce resting B cells proliferation and differentiation into Ig secreting plasma cells in the presence of IL-4 (Nakanishi et al, 1996; Rush and Hodgkin, 2001; Linterman and Beaton et al, 2010).

B cells also express several TLRs and TLR, especially TLR4 and TLR9 signals, can induce polyclonal B cell activation and function (Bernasconi et al, 2003; Bourke, 2003; Ruprecht and Lanzavecchia, 2006; Richard et al, 2008). Human and mice B cells respond to TLR4 and TLR9 signals differently. Murine B cells stimulation with LPS induces polyclonal expansion, antibody isotype switching and differentiation to plasma cells (PCs), while stimulation with CPG can induce only proliferation but less IgG production (Bernasconi et al, 2003; Lanzavecchia and Sallusto, 2007; Richard et al, 2008). TLR9 is expressed at high levels in naive and memory human B cells compared to that in murine (Bourke et al, 2003; Ruprecht and Lanzavecchia, 2006); and CPG is an excellent agent to induce human B cell proliferation and antibody production (Krieg, 2000; Ruprecht and Lanzavecchia, 2006). CPG/TLR9 signals also enhance APC function of B cell by upregulating surface CD40, CD80 and MHC-II expression (Jiang and Lederman et al, 2007).

To date, it is unclear whether IL-37a is bioactive and whether IL-37a and IL-37b isoforms are different in expression and function. Based on the structural study in Chapter 3 and the similarity between IL-37a and b in the IL-1-like receptor-binding domain, I hypothesised that IL-37a is bioactive and can also bind to and signal via the same receptor. However, the two isoforms also possess different N-terminal sequence that may offer them unique biological features in cellular location and function. Thus the main aims of this chapter are:

- 1- To investigate the induction and regulation of IL-37 isoforms and receptor expression in human cells.
- 2- Determine the bioactivity of endogenous and exogenous IL-37a in the regulation of human cell functions.
- 3- Compare the similarity and difference between IL-37a and IL-37b isoforms in the regulation of immune cell functions.

4.2 Results

4.2.1 IL-37 isoform expressions levels are differently regulated in human monocytes.

To understand the induction and regulation of IL-37 isoforms in immune cells, we sought to investigate IL-37 isoform expression in human monocyte THP-1 cells. Monocytes were cultured in RPMI medium; and stimulated with or without different doses of LPS for 16 hours. Cells were harvested and the IL-37 isoform expressions determined by qPCR. We found that different doses of LPS had different effect on the expression of IL37 isoforms. 100 ng/ml of LPS markedly induced the expression of all isoforms, compared to the controls (Fig 4.1). The IL-37a, IL-37c and IL-37e expressions were higher than IL-37b and IL-37d. At the higher dose (200 ng/ml), LPS mainly induced the expression of IL-37d, not the other isoforms.

Next, we investigated the time effect of LPS on the induction of IL-37 isoforms expression. THP-1 cells were stimulated with fixed dose of LPS and the IL-37 isoform expressions were determined by qPCR over the times indicated. As shown in (Fig 4.2); the kinetics of induction of the isoform expressions were

varied; the expression of IL-37a, c, d and e reached to peak at 16 hours after LPS stimulation and then declined. In contrast, IL-37b was induced in a time dependent manner, at 24 hours when all other isoform expressions were declined, the expression of IL-37b still went up (Fig 4.2).

These results suggest that the expression of IL-37 isoforms is differently regulated and IL-37a is one of the highly induced isoforms.

4.2.2 IL-37a is an immune suppressive cytokine.

I next investigated if the IL-37a is bioactive and whether it has same or different bioactivity in immune cells compared to IL-37b.

In order to understand the function of the endogenous IL-37a, we studied its function in human monocyte in vitro. We firstly knockdown the RNA of IL-37a or total IL-37 isoforms as well by using specific siRNAs that target exon 3 for IL-37a and the common exon 5 for all the IL-37 isoforms respectively. The human monocyte cell line THP1 cells were transduced with the specific and control siRNA for 48 h and then the cells were stimulated with LPS for 6 hours followed by the determination of IL-37 isoforms, TNF- α and IL-1 α expression by qPCR. As shown in (Fig 4.3), knockdown of IL-37a alone significantly enhanced the expression of LPS-stimulated inflammatory cytokine TNF- α , which was further enhanced by the knockdown of total IL-37 isoforms. The knockdown of both IL-37a and total IL-37 also significantly elevated the expression of LPS-induced IL-1 α with the similar effect. These siRNAs also selectively abolished the expression of IL-37a and total IL-37 (Egle Katkeviciute, data not shown). Thus, this result suggests that endogenous IL-37a is bioactive and may serve as an immunosuppressive cytokine.

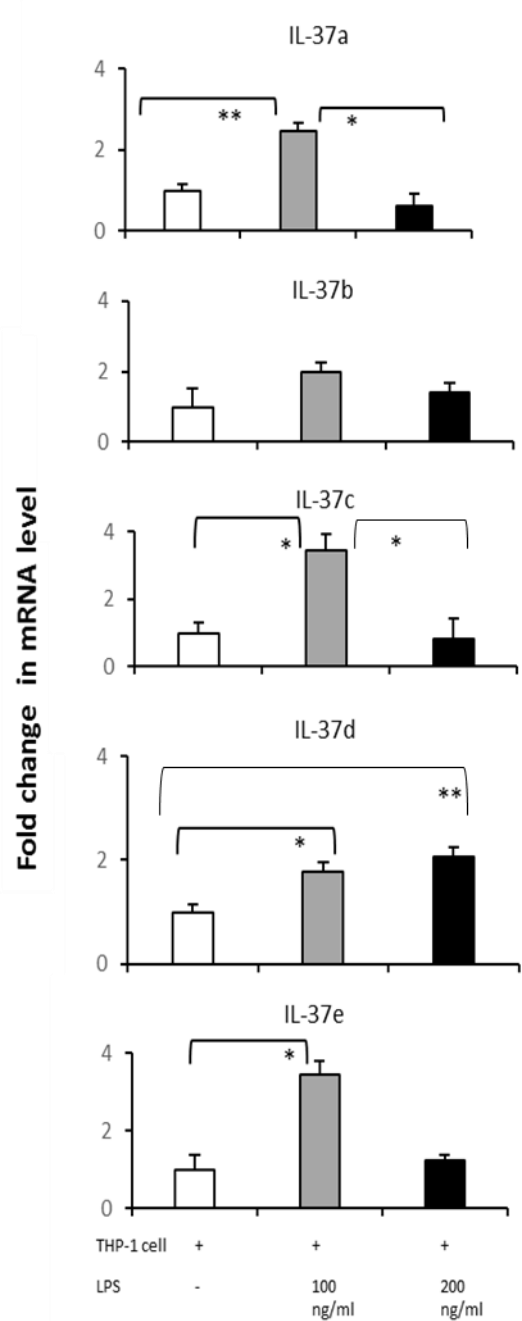


Figure 4.1 Dose-dependent effect of LPS in the induction of IL-37 isoforms expression in THP-1 cells.

THP-1 cells ($10^6/\text{ml}$) were stimulated with different doses of LPS as indicated for 16 hours then the RNA extracted for qPCR with specific primers for all the IL-37 isoforms. Data are mean \pm SD, * $P < 0.05$ compared to controls, which is THP-1, cells alone or treated with LPS. This figure represents three independent experiment.

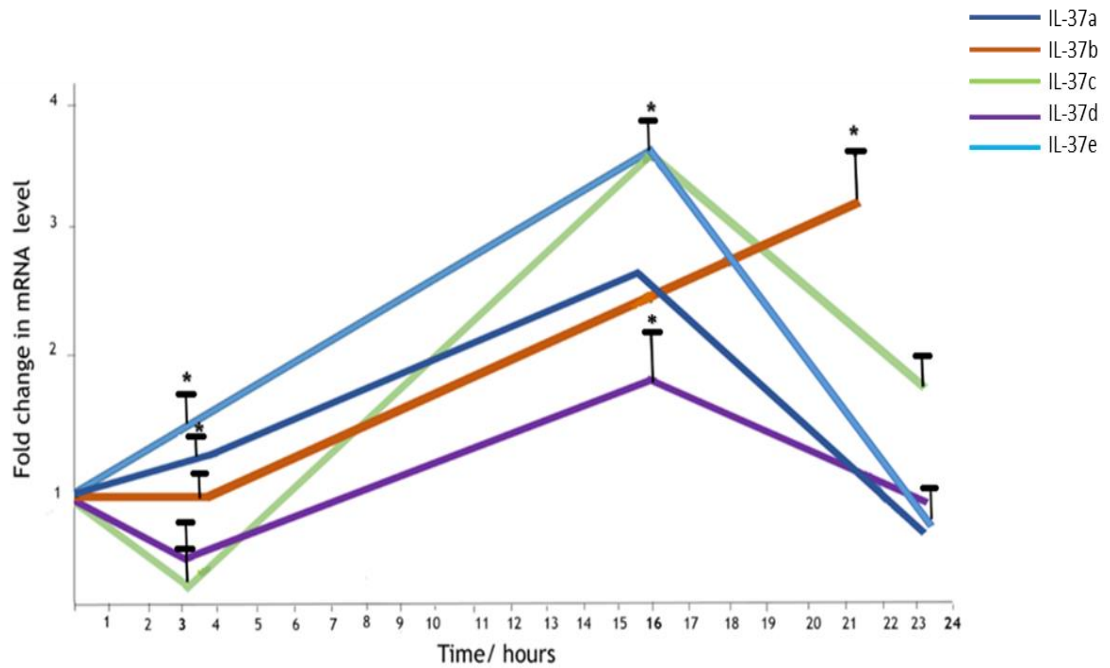


Figure 4.2 Time effect of LPS on the induction of IL-37 isoform expression

THP-1 cells ($10^6/\text{ml}$) were stimulated with 100 ng/ml of LPS, the RNA were extracted and the expression levels of IL-37 isoforms monitored at 3, 16 and 24 hours by qPCR using specific primers. Data are mean \pm SD, * $P < 0.05$ compared to controls which is THP-1, cells alone or treated with LPS. This figure represents three independent experiment.

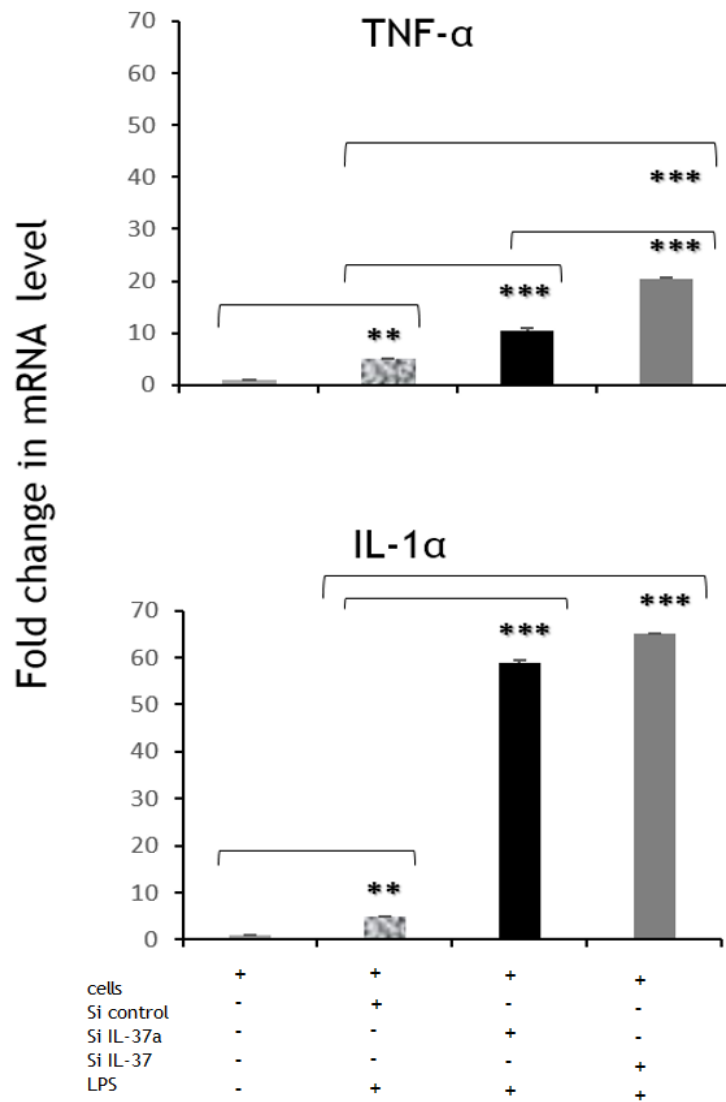


Figure 4.3 Knockdown of IL-37a and total IL-37 by specific siRNA increases LPS stimulated inflammatory cytokines production in THP-1 cells.

The RNA of IL-37a and total IL-37 isoforms in THP1 cells were knocked down first using specific siRNAs; the cells ($10^6/\text{ml}$) was then stimulated with LPS for 12 h; and the expression of IL-1 α and TNF- α was determined by qPCR. Data are mean \pm SD, ** $P < 0.01$, and *** $P < 0.001$. This figure represents two independent experiment.

In chapter 3, I purified FL-IL-37a and IL-37b proteins by Ni column and gel filtration. I next tested their bioactivity in the mouse macrophage (RAW 264.7 cell line) which lack IL-37 gene expression but possess both receptors (IL-18Ra and IL-1R8) for signalling of IL-37 (Bulau et al, 2014; Li et al, 2015). This cell line has been used to test IL-37b activity (Bulau et al, 2014). RAW 264.7 cells cultured in DMEM medium were stimulated with LPS in the presence or absence of different doses of recombinant IL-37a and IL-37b. These proteins were purified by Ni-NTA column (Fig 4.4 A and B) or by both Ni chromatography and gel filtration (Fig 4.4 C and D) as described in Chapter 3; the cells were then harvested 24 hours after the culture and IL-6 levels in culture supernatants were measured using ELISA. I found that both rIL-37a and rIL-37b proteins purified by Ni column alone were sufficient to suppress the LPS-induced IL-6 production in macrophages, and IL-37a was more suppressive than IL-37b in this condition (Fig 4.4 A and B). The inhibitory effect of both proteins on IL-6 production was dose-dependent, with the lower doses (50-25 ng/ml) more suppressive than the higher ones. This is consistent with the report that IL-37b can form homodimer at higher concentration and homodimer is less suppressive than monomer in inflammatory cytokine production (Ellisdon et al, 2017). Compared to the Ni column purified proteins, I also found that the inhibitory capacity of IL-37a and IL-37b proteins purified by both Nickeled-column and gel filtration was more pronounced, and again, IL-37a was more suppressive compared to IL-37b (Fig 4.4 C and D). To confirm the differences between IL-37a and IL-37b in bioactivity, we next compared the ability of both proteins in the suppression of IL-6 production in LPS-stimulated macrophage. As shown in (Fig 4.5). IL-37a was more suppressive than IL-37b in IL-6 production in macrophages in a dose-dependent manner.

I further assessed the regulatory effect of IL-37a on other key cytokines production in LPS-stimulated monocytes. I found that IL-37a could also suppress LPS-induced expression of TNF- α and IL-1 α , the important inflammatory cytokines; however, it had no effect on the inhibition of regulatory cytokine IL-10 expression in the condition (Fig 4.6). Together, all the data demonstrated for the first time that both the endogenous and recombinant IL-37a are functional and able to suppress LPS-induced cytokine production in human monocyte and murine macrophages, the important innate immune cells.

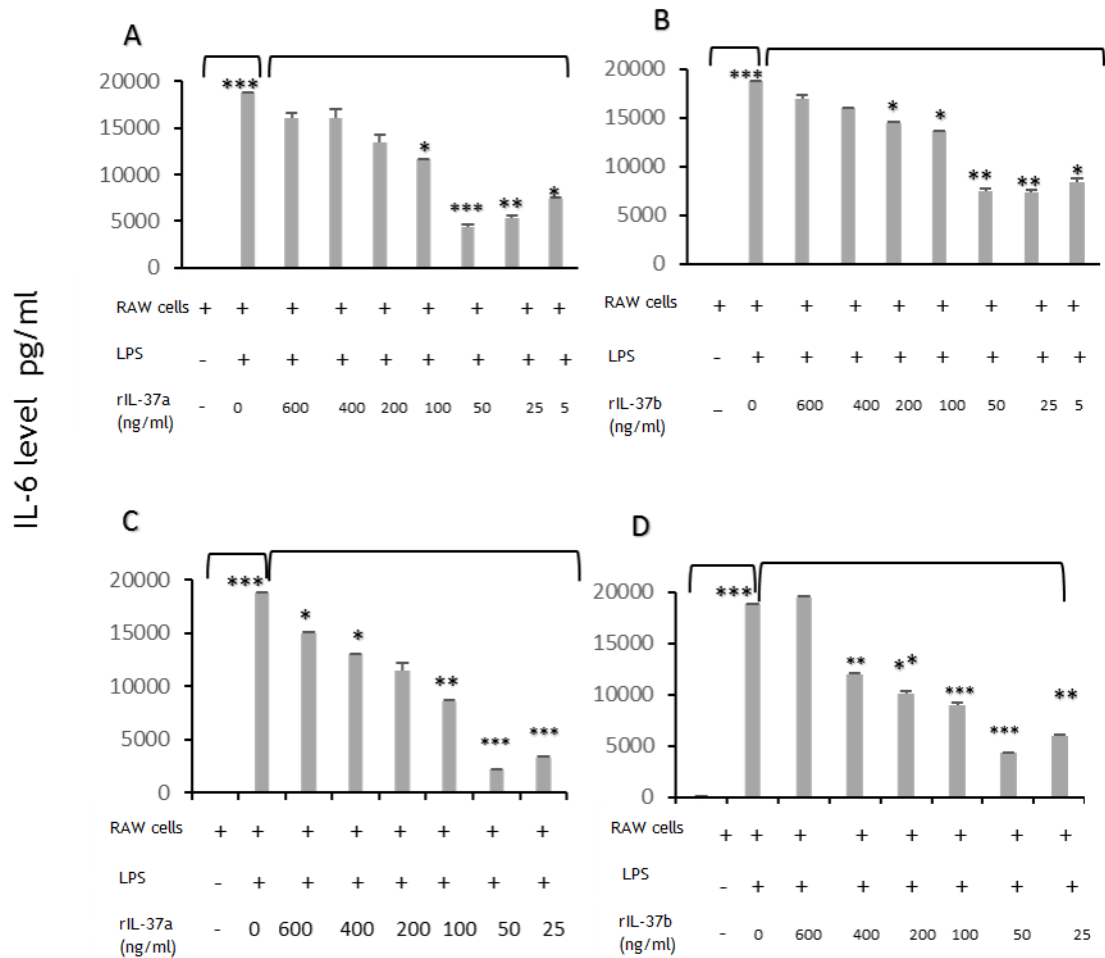


Figure 4.4 Bioactivity of recombinant IL-37a and IL-37b in LPS-stimulated murine macrophage.

Murine RAW 264.7 cells (10^6 /ml) were stimulated with LPS (100 ng/ml) and treated with different dose of rIL-37a and rIL-37b purified by different columns. Cells were harvested after 24 hour culture, and ELISA used to measure IL-6 levels in cells supernatant. (A and B) represent the activity of rIL-37a and rIL-37b purified by Nicked-column. (C and D) are the bioactivity of rIL-37 after gel filtration and LPS removal. Data are mean \pm SD, * P < 0.05, ** P < 0.01, and *** P < 0.001 compared to control (RAW 264.7 cells alone or treated with LPS). This figure represents two independent experiment.

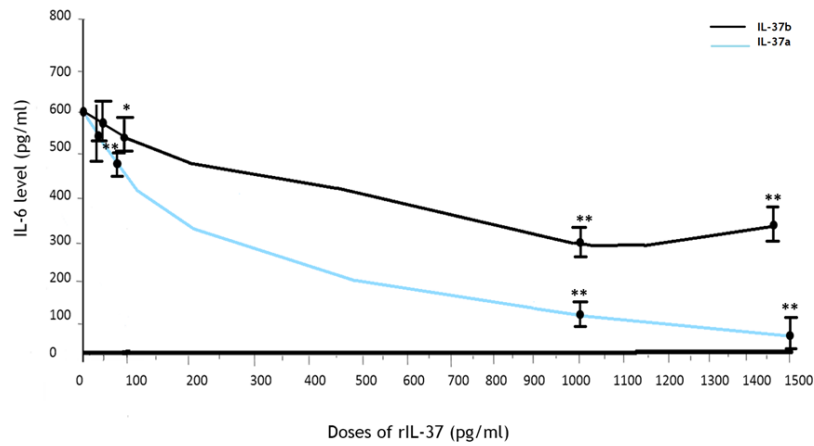


Figure 4.5 Comparison between recombinant IL-37a and IL-37b bioactivity in murine macrophage RAW264.7.

Murine RAW cells (10^6 /ml) were stimulated with LPS (100 ng/ml) and treated with different doses of rIL-37a and rIL-37b for 24 hours; and IL-6 level in culture supernatant was measured by ELISA. Data are mean \pm SD, * P < 0.05 and ** P < 0.01 compared to control (RAW264.7 treated with LPS). This figure represents three independent experiment.

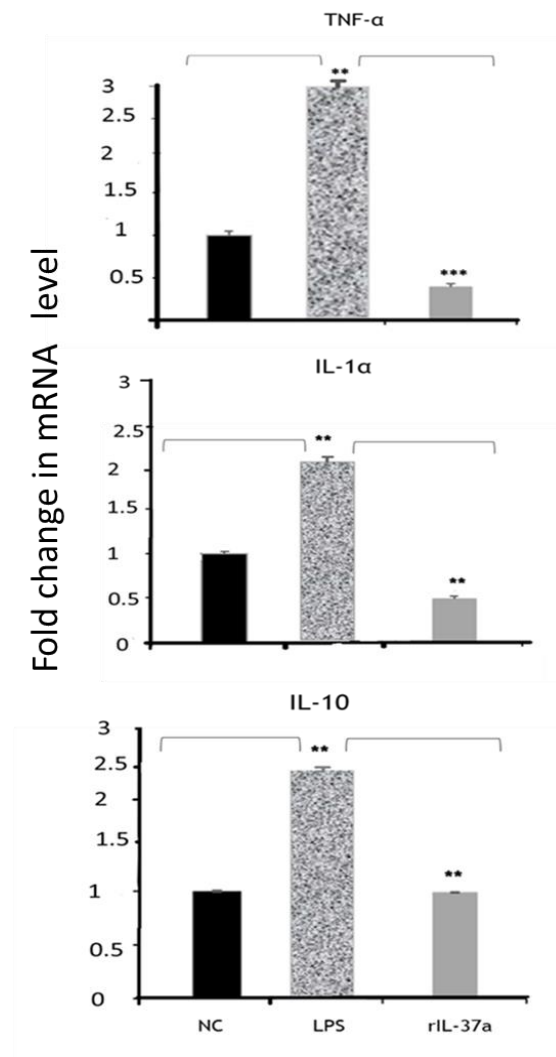


Figure 4.6 Effect of rIL-37a on the regulation of cytokine expression.

THP-1 cells ($10^6/\text{ml}$) were stimulated with LPS (100 ng/ml), in the presence of rIL-37a (50 ng/ml); the cells were harvested for RNA extraction after 16 hours culture. TNF- α , IL-1 α and IL-10 expressions were determined by qPCR. Data are mean \pm SD; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to control (NC: THP-1 cells only) or LPS treated THP-1 cells. This figure represents three independent experiment.

4.2.3 The regulatory effect of IL-37a and IL-37b on murine B cell function

4.2.3.1 IL-37a and IL-37b increase antibody production in B cells

After finding that IL-37a and IL-37b isoforms suppress pro-inflammatory cytokines production in innate immune cells, we decided next to investigate the role of IL-37 in adaptive immune cells. While the effect of IL-37b on the regulation of T cell function has been studied (Shuai et al, 2015; Wang et al, 2016), the role of IL-37a and IL-37b in B cells is unknown.

B cells represent one of the major arms of adaptive immunity and have three functions; they serve as professional APC, producing cytokine; but their main function is antibody production in humoral immune responses. Therefore, we sought to investigate the effect of IL-37 on B cell function; the first step was to check the effect of IL-37a and IL-37b on antibody production in murine B cells. Murine CD19⁺ B cells were isolated from spleenocytes by MACS using anti-CD19 antibody conjugated on magnetic beads for positive selection (Abts et al, 1989).

CD40 ligand on T cells plays a co-stimulatory role in B cell activation and antibody production by interacting with CD40 receptor on B cells and triggering signalling (Parker, 1993; Smith et al, 2000; Elgueta et al, 2009; Zotos et al, 2012). We then assessed the regulatory effect of IL-37 on the CD40-mediated B cell activation and antibody production. The B cells were co-cultured with 3T3 cells that over-express transgenic CD40 ligand, and then treated with 50 ng/ml of rIL-37a and rIL-37b for 3, 4 and 7 days. Supernatants were collected for the detection of IgG level in the cultures by ELISA. As reported, CD40 signals significantly enhanced B cell IgG production from 3 days culture (Fig 4.7 A.C). Unexpectedly, rIL-37a further increased the IgG production in CD40-activated B cells 7 days after the culture (Fig 4.7C). This was also the case in the rIL-37b treated B cells (Fig 4.8). Furthermore, rIL-37b was more effective than rIL-37a in the enhancement of B-cell IgG production (Fig 4.7 and 4.8).

I sought next to confirm the phenomenon by using B cells isolated from the human IL-37a transgenic (IL-37a-tg) mice which constantly express IL-37a (Dr Xu, unpublished result). CD19⁺ B-cells were isolated from wt and IL-37a transgenic C57BL/6 mice as above and co-cultured with CD40L expressing 3T3 cells for 3, 4

and 7 days as before (Fig 4.7 and 4.8). Consistent with the results with rIL-37 protein, the B cells from IL-37a transgenic mice also showed an increased IgG production compared to the wt B cell control, 7 days after the culture (Fig 4.9)

TLR signals also play an important role in B-cell activation and antibody production, in particular TLR4 and TLR9 signals (Jiang et al, 2007; Quintana et al, 2008; Boeglin et al, 2011). I then determined whether IL-37a and IL-37b can also potentiate the TLR4 agonist, LPS-mediated antibody production in B cells. CD19⁺B cells were isolated from spleenocytes of C57BL/6 mice and stimulated with LPS (10 ug/ml) in the presence or absence of rIL-37. As seen in Fig 4.10 and 4.11, both rIL-37a and rIL-37b increased LPS-induced IgG production in B cells. The effect was more pronounced in IL-37b than IL-37a treated B cells (Fig 4.10 and 4.11).

Furthermore, B cells from IL-37a-tg mice also markedly enhanced IgG production in the LPS-stimulated B cells from 3 days after the culture (Fig 4.12). At day 7, the antibody level in the tg cells were about 5 times higher than that in wt control cells. Thus, both IL-37a and IL-37b are able to promote antibody production in the CD40 or TLR activated B cells.

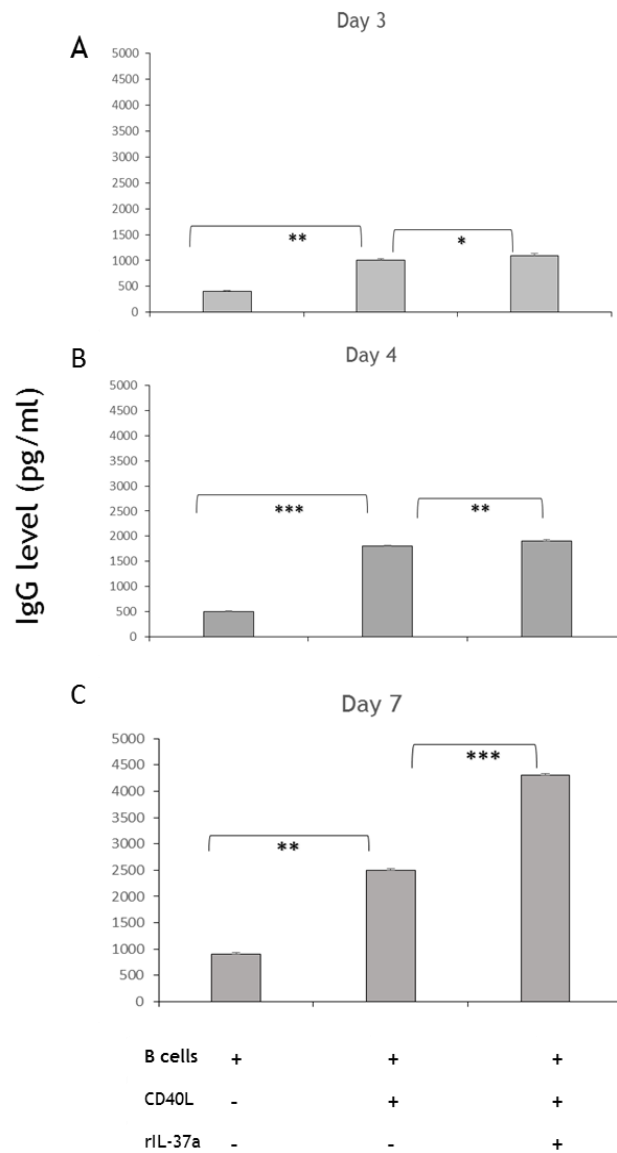


Figure 4.7 IL-37a enhanced IgG production in CD40-stimulated B-cell.

Wild type CD 19 murine B cells (10^6 cell/ ml) were cultured with 3T3 (15×10^4 cells/ml), and treated with 50 ng/ml of rIL-37a, and incubated for 3, 4, and 7 days, IgG levels in the culture supernatant was measured using ELISA. A, B, and C are IgG levels at days 3, 4, and 7. Data are mean \pm SD; * $p \leq 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control (CD19⁺ cells only) or to CD40 treated cells. This figure represents five independent experiment.

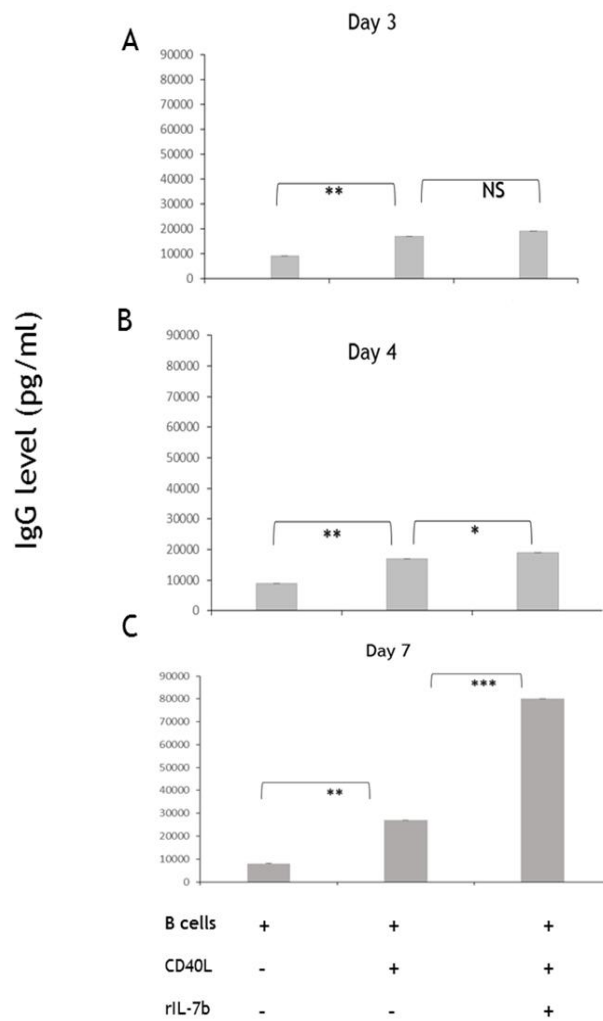


Figure 4.8 IL-37b enhanced IgG production in CD40 stimulated B-cell.

Wild type CD19⁺ murine B cells (10^6 cell/ ml) were cultured with 3T3 cells (15×10^4 cells/ ml), and treated with 50 ng/ml of recombinant IL-37b, cells were incubated for 3, 4, and 7 days. ELISA used to measure IgG levels in the culture supernatant. The experiment was repeated more than five times. A, B, and C are IgG levels at days 3, 4, and 7. Data are mean \pm SD; NS represents non-significant, and * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control or CD40L treated cells. This figure represents five independent experiment.

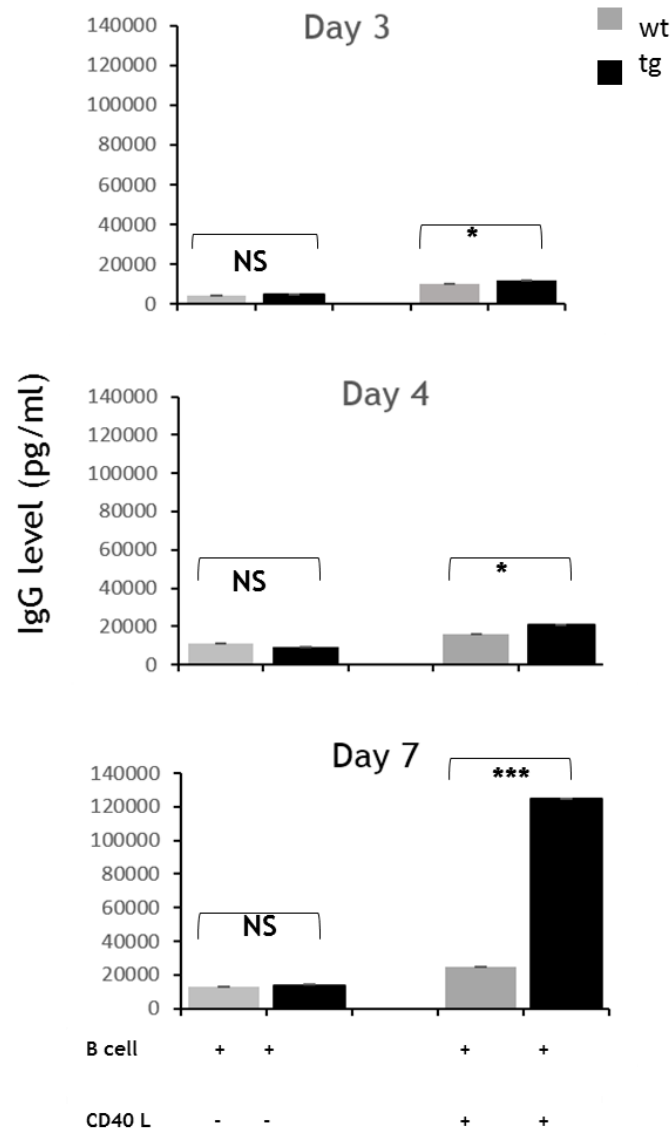


Figure 4.9 IL-37a-tg mice elevate IgG production in CD40-stimulated B-cell compared to wt cells.

CD 19⁺ murine B cells (10^6 cell/ml) from wt and tg-mice were cultured with 3T3 for 3, 4, and 7 days. IgG level in the culture supernatant measured by ELISA. Experiment was repeated more than 5-8 times. A, B, and C are IgG levels at days 3, 4, and 7. Data are mean \pm SD; NS represents non-significant, and *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control or CD40. This figure represents five independent experiment.

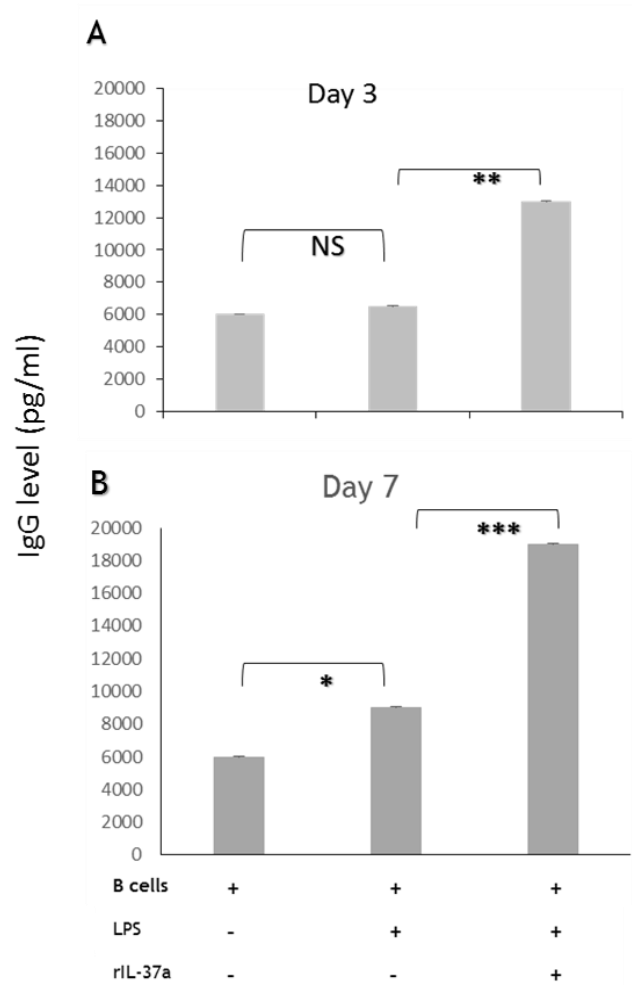


Figure 4.10 IL-37a enhance IgG production in LPS-stimulated murine B-cell.

Wild type CD19⁺ murine B cells was cultured (10⁶ cell/ml) in RPMI, and treated with 10 µg/ml of LPS, followed by 50 ng/ml rIL-37a for 3 and 7 days. IgG level measured from the supernatant of the cultured cells by ELISA. A and B are IgG levels at different days. Data are mean ± SD; NS represents non-significant, and *P < 0.05, **P < 0.01, and ***P < 0.001 compared to control or LPS CD19⁺ treated cells. This figure represents seven independent experiment.

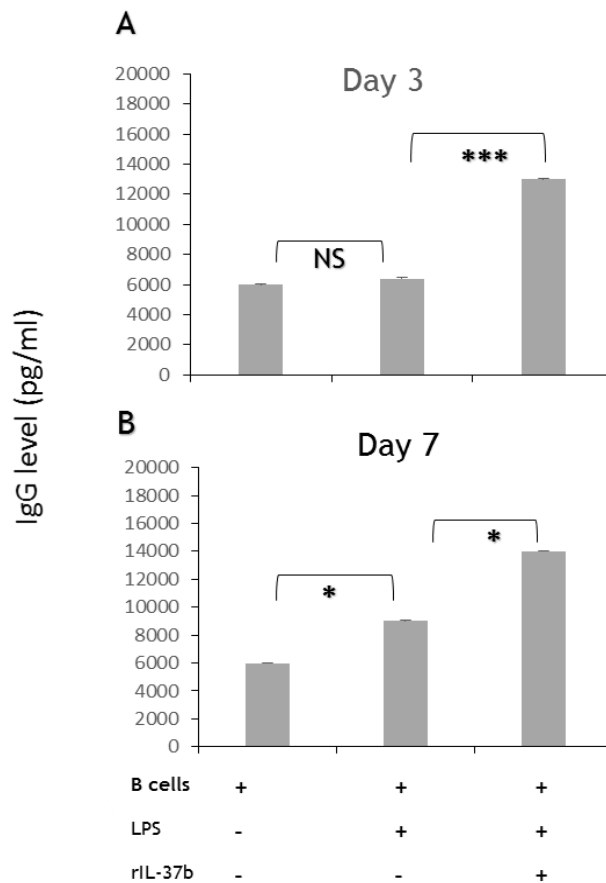


Figure 4.11 IL-37b enhance IgG production in LPS-stimulated murine B-cell.

Wild type CD19⁺ murine B cells were cultured (10^6 cell/ml) in RPMI, and treated with 10 ug/ml of LPS, followed by 50 ng/ml of rIL-37b for 3 and 7 days. IgG level measured from the supernatant of the cultured cells by ELISA. A and B are IgG levels at different days. Data are mean \pm SD; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control or LPS treated cells. This figure represents three independent experiment.

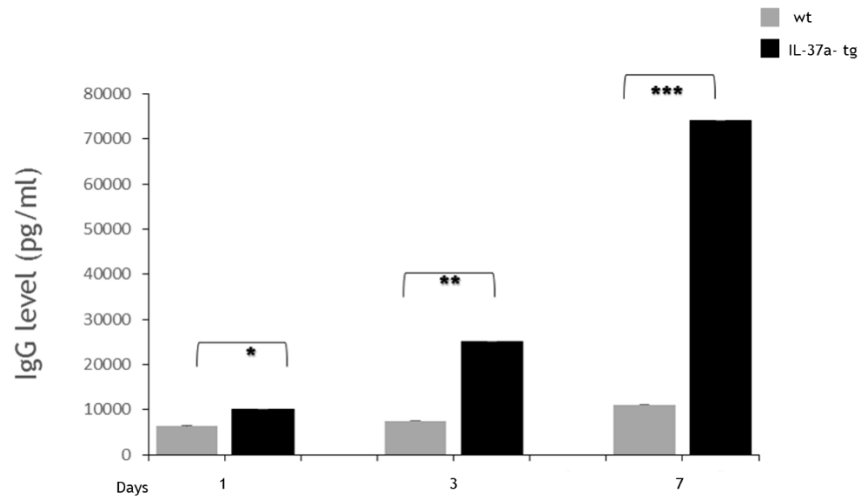


Figure 4.12 IL-37a-tg mice increase IgG in LPS-stimulated murine B-cells.

Wild type and tg- CD19⁺ murine B cells were cultured (10^6 cell/ml), treated with 10 ug/ml of LPS for 3, 4, and 7 days. IgG level were measured from supernatant of the cultured cells by ELISA. Data are mean \pm SD; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to wild type cells stimulated with LPS. This figure represents three independent experiment.

4.2.3.2 IL-37 promotes B-cell proliferation

I next studied the possible mechanism by which IL-37 enhances B cell antibody production. First, I assessed the possibility that IL-37 may promote antibody production by inducing B-cell proliferation. The CD19⁺ B cells were isolated from spleenocytes of wt and IL-37a-tg C57BL/6 mice and cultured with or without LPS (10 ug/ml) as before for 2, 4 and 7 days. B-cell proliferation was determined by Brdu cell proliferation assay as described (Löfdahl et al, 2018). As illustrated in the (Fig 4.13), without LPS, IL-37a-tg cells exhibited a slight but significantly enhanced cellular proliferation compared to that in the wt cells. While LPS alone had little effect on the B-cell proliferation in wt group in this condition, it markedly enhanced cell proliferation in IL-37a-tg cells. The rIL-37a and rIL-37b also enhanced proliferation in murine B cell isolated from wt mice with or without LPS in vitro (data not shown). This result suggests that IL-37a can promote B cell proliferation, alone or with LPS.

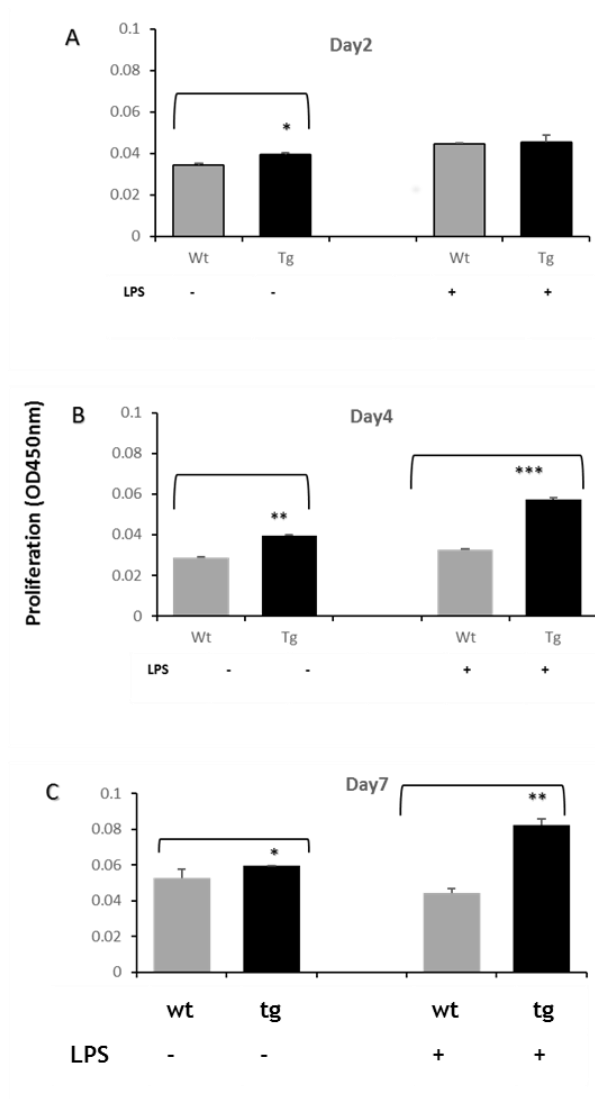


Figure 4.13 IL-37a transgenic B cells enhance proliferation compared to wild type B cells.

CD19⁺ murine B cells from wt and tg- mice were stimulated with or without LPS (10 ug/ml). Brdu 0.1 % (v/v) was added to the cell cultures at days 1, 3, and 6, incubated for 15 hours from the addition, and the cells harvested for proliferation measurement. Proliferation were measured at 450 nm. Data are mean \pm SD, *P< 0.05, **P< 0.01, and ***P< 0.001 compared to control (wt cells treated with LPS). This figure represents three independent experiment.

4.2.3.3 IL-37a regulates cytokine and signalling molecule expressions in activated murine B cells

B-cell activation and antibody production process is regulated by signalling pathways elucidated by BCR, co-stimulatory molecules and cytokines (De Silva and Klein, 2015; Wu et al, 2016). Cytokines play different but crucial roles in B cell differentiation, antibody class switching and proliferation, including IL-10, TGF- β , IFN γ and IL-13 (Jurado et al, 1989; Cocks et al, 1993; Abed et al, 1994; Defrance, 1994; Gros et al, 2008). I therefore investigated next whether IL-37 enhances B cell function by the regulation of different cytokine expression. CD19⁺B cells were isolated from wt and IL37a-tg spleenocytes by MACS. The B cells (10^6 /ml) were cultured with or without 10 μ g/ml of LPS for different times and cytokine gene expression determined by qPCR. First, we checked IL-10, which can enhance B cell antibody production by preventing B cell apoptosis (Gary-Gouy, 2002). I found that 1 day after culture; IL-10 expression was markedly enhanced in tg-mice, compared to that in wt cells (Fig 4.14). However, the IL-10 expression was significantly reduced after 3 and 7 day cultures compared to wt control cells. The expression of antibody isotype class switching cytokines (TGF- β , IFN- γ , IL-13) in LPS-stimulated B cells was also determined by qPCR (Fig 4.15). The expression of TGF- β was markedly enhanced (245 fold) in LPS-stimulated IL37a-tg B cells compared to wt cells. The expression levels of IL-13 were also elevated, while the IFN γ was significantly reduced in IL37a-tg cells compared to wt cells (Fig 4.15).

We also checked the expression of another three genes, phosphatidylinositol-3, 4, 5-trisphosphate 5-phosphatase 1 (*Inpp5d*) or called SHIP (SH2 domain-containing inositol 5' phosphatase), tyrosine-protein kinase (Lyn), and tumor necrosis factor ligand superfamily member 13 (*Tnfsf13*) or (April). SHIP knock out (SHIP^{-/-}) mice display high serum IgG (Helgason et al, 2000; Maeda and Mehta et al, 2010), referring that SHIP is critical in the limitation of B cell responsiveness to antigen stimulation. Lyn can enhance or reduce antigen B cell receptor-mediated signals (Yamanashi et al, 1993; Kurosaki et al, 1994; Sidorenko et al, 1995; Tezuka et al, 1996; Wang, 1996; Chan et al, 1997; Yamanashi et al, 1997). Lyn deficiency causes lupus-like autoimmune disease and B cell hyperactivity (Lamagna et al, 2013). April (A proliferation-inducing

ligand), plays a key role in peripheral B survival, maturation, and differentiation. April^{-/-} mice have normal B lymphocyte development and proliferation in vitro, but high level of IgG (Castigli et al, 2004). We noticed that B-cells from IL-37a-tg mice significantly decreased the expression of SHIP with or without LPS, but increased expression of Lyn after LPS stimulation (Fig 4.16). The expression level of April was similar between IL-37a-tg and wt B cells in the presence or absence of LPS.

These results suggest that IL-37-induced B cell antibody production and proliferation in mice may also be attributed, in part, to its ability to induce and regulate the expression of key cytokines and signalling molecules.

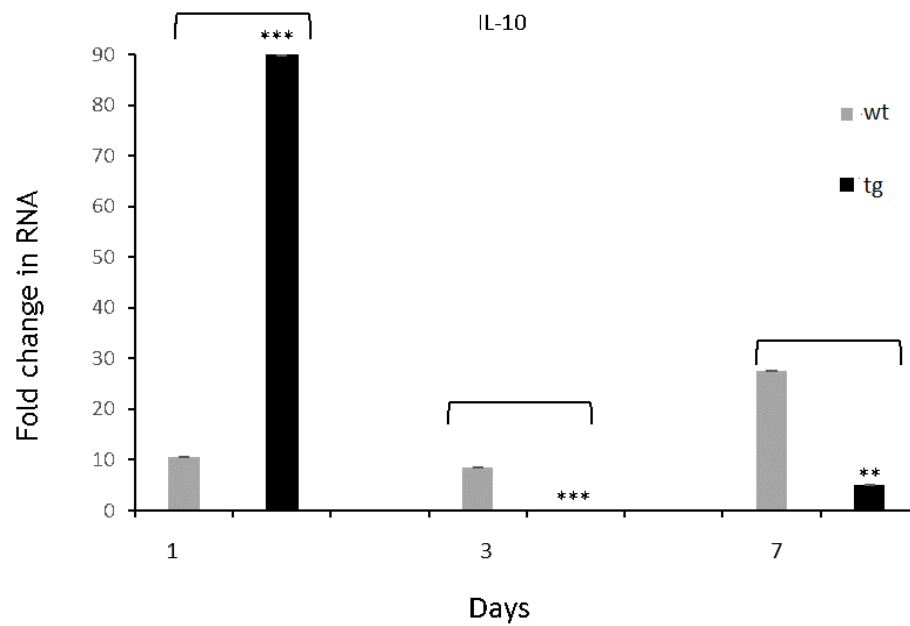


Figure 4.14 IL-37a-tg B cells alter IL-10 expression.

CD 19⁺ cells (10⁶/ml) from IL-37a-tg, and wt mice were cultured and stimulated with 10 µg/ml of LPS for 1, 3 and 7 days; the RNA extracted and qPCR performed to determine the IL-10 level. Data are mean ± SD, **P < 0.01 and ***P < 0.001 compared to control or (wild type LPS stimulated CD19⁺). Both wt and tg stimulated with LPS are normalised to non-treated wt and tg cells respectively, thus the comparison here is only between treated wt and tg-cells. The figure represents three independent experiment.

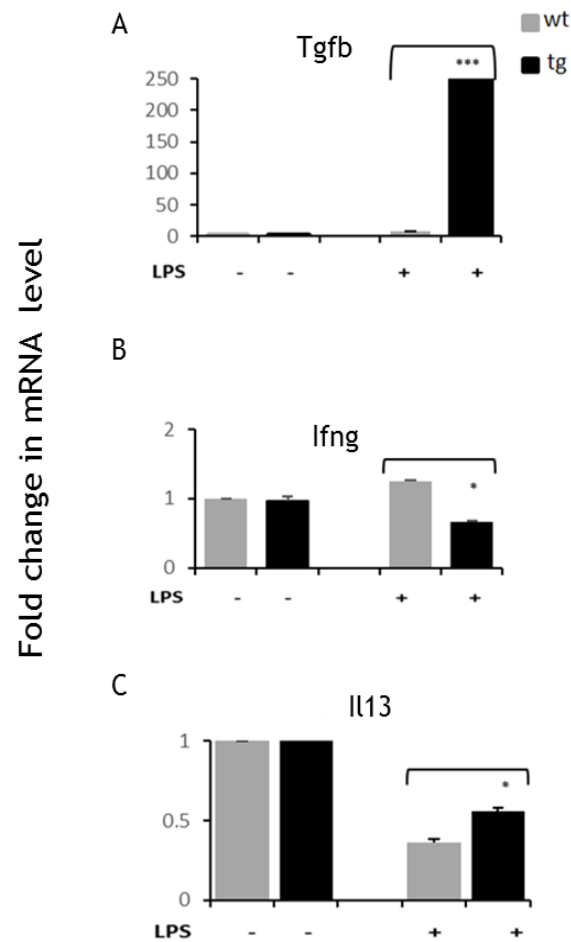


Figure 4.15 IL-37a-tg B cells alter antibody isotype switching cytokine expression upon stimulation with LPS compared to wt mice.

CD19⁺ cells (10⁶/ml) from IL-37a-tg and wt mice were cultured and stimulated with LPS (10 ug/ml) for 3 days; RNA extracted and qPCR performed to detect the expression of A) Tgfb, B) Ifng and C) Il13. Data are mean \pm SD, *P < 0.05 and ***P < 0.001 compared to control (wild type). The figure represents three independent experiment.

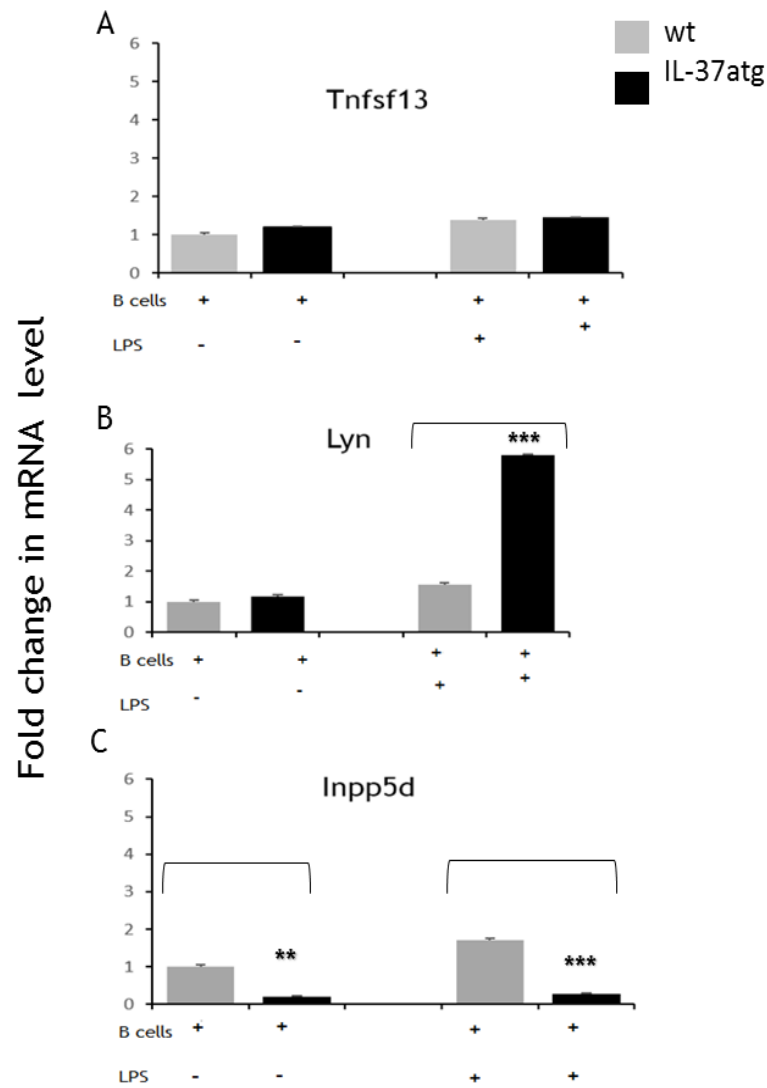


Figure 4.16 Role of IL-37a in the regulation of the expression of Tnfsf13, Lyn and Inpp5d in transgenic B-cells compared to wt controls.

CD19⁺ cells (10⁶/ml) from IL-37a-tg and wt mice were cultured and stimulated with LPS (10ug/ml) for 3 days; RNA was extracted and qPCR performed to detect the expression of: A) Tnfsf13 or April; B) Lyn and C) Inpp5d or SHIP. Data are mean \pm SD, **P < 0.01 and ***P < 0.001 compared to control (wild type). The figure represents three independent experiments.

4.2.4 Effect of recombinant IL-37a and IL-37b on human B cells

4.2.4.1 The effect of rIL-37a and rIL-37b on human B cells antibody production

Since the most dramatic effect of IL-37 in murine B cells is the promotion of antibody production, we next sought to confirm the effect of IL-37 in human B cells.

It is known that while both CpG and LPS can enhance antibody production in B cells, B-cells from human and mouse are different in their response to the two TLR agonists (Richard et al, 2008). Human B cells response to CpG but not LPS; while murine B cells prefer LPS stimulation (Lutzker et al, 1988; Severinson et al, 1990; Berkeredjian-Ding et al, 2002; Hornung et al, 2002; Wangner et al, 2004). Therefore, Cytosine-phosphate-guanine class B or (CpG (ODN 2007)), was used to study antibody induction in human B cells (Malaspina et al, 2008)

The CD19⁺ cells were purified from PBMC of buffy-coat human blood cells using MACS by positive selection (Chapter 2). The purity of B cells was about 98% of total cells, as determined by flow cytometry using anti-CD20 antibody (Fig 4.17).

The B cells were cultured in complete RPMI medium and stimulated with CpG (3 ug/ml) in the presence or absence of different doses of rIL-37a and rIL-37b (Fig 4.18), for 4, 7 and 9 days. The cells were harvested for gene expression by qPCR, and the supernatants for IgG measurement using ELISA. The ELISA results revealed that, as expected, CpG induced B cell antibody production from day 4, however, 50 pg/ml of IL-37a and IL-37b only enhanced CpG-induced IgG production at 4 days after culture, not at day 7 and day 9 (Fig 4.18). Increasing the doses of IL-37 from 50 pg and 50 ng failed to further enhance the antibody production at all the tested time points (Fig 4.18).

To understand if the weak efficacy of IL-37 in human B cell antibody production is due to the lost bioactivity of the recombinant proteins or due to IL-37 having different effect between human and murine B cells, I then assessed the cytokine expression level in the cultured B cells, including the Th1, Th2 and Treg cytokines. I found that IL-37a alone significantly inhibited the expression of Th1 cytokines, IL-12, IFN β and TNF- α , but not IFN γ , compared to cell only control

(Fig 4.19) IL-37b alone significantly decreased IFN γ , IL-12a but not the IFN β and TNF- α . CpG alone downregulated all 4 cytokine at this condition, compared to the cell control. IL-37a and CpG further down regulated all the cytokines, except the IFN γ compared to CpG alone. IL-37b and CpG further suppressed all 4 cytokines compared to CpG control (Fig 4.19).

I further checked the regulatory effect of IL-37a and IL-37b on the expression of Th2 (IL-13, IL-6) and Treg (TGF- β and IL-10) cytokines in human B cells with or without the stimulation of CpG. As shown in the (Fig 4.20), IL-37a alone significantly reduced the expression of TGF- β , IL-10 and IL-6, but enhanced the expression of IL-13, compared to cell alone control. IL-37b alone had no effect on the expression of TGF- β and IL-10, while, as IL-37a, it also enhanced IL-13 but reduced IL-6 expression. CpG alone markedly increased TGF- β and IL-10 but decreased IL-13 and IL-6 expression compared to control. While CpG together with IL-37a and IL-37b further enhanced TGF- β and IL-10, but reduced, IL-13 and IL-6 expression compared to CpG and cell alone controls (Fig 4.20).

Thus, the recombinant IL-37 proteins were bioactive and could potentiate CpG-mediated regulatory effect on Th1, Th2 and Treg cytokine expressions in B cells. Furthermore, IL-37a and IL-37b alone can differently influence these cytokine expressions in human B cells.

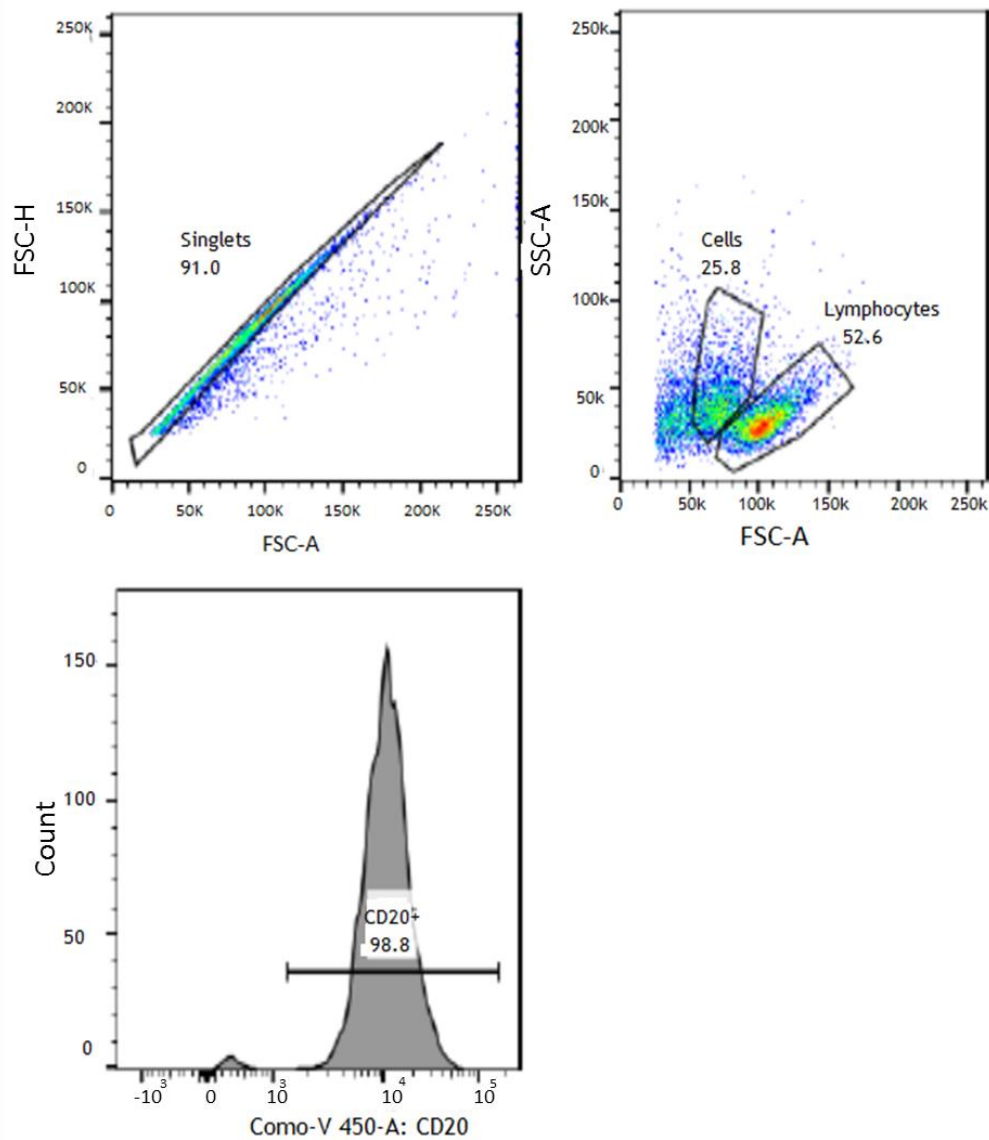


Figure 44.17 The purity of isolated human B cells determined by flow cytometry

Human CD19⁺ B cells were isolated from PBMC by MACS using anti-CD19 beads. The B cells were then labelled with anti-CD20 antibody conjugated with FITC to check the purity of the isolated B cells by flow cytometry. The figure shows B cells purity which is 98.8% of.

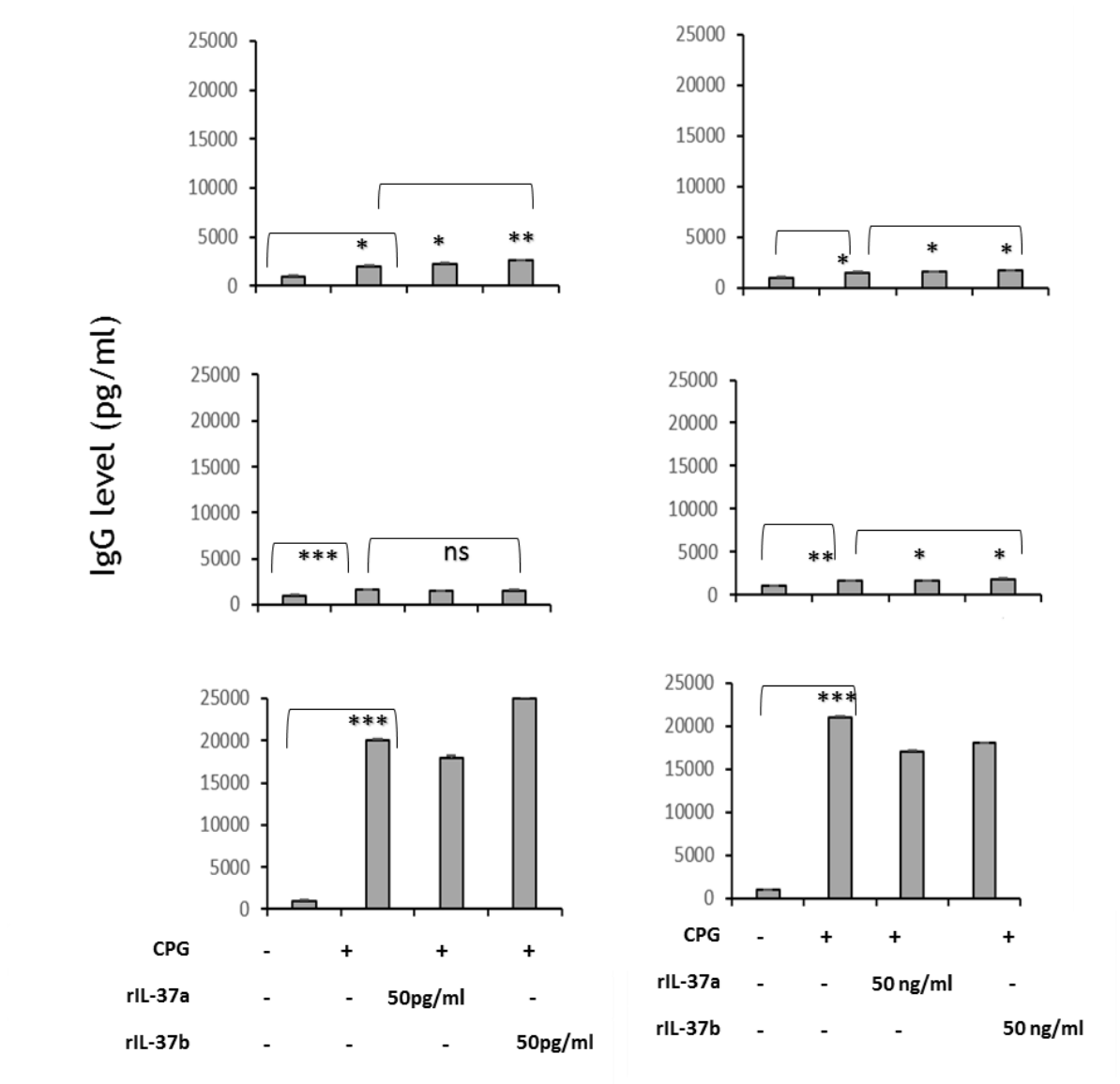


Figure 4.18 The effect of IL-37a and IL-37b on IgG production in human B-cells.

Human CD19⁺ B cells were isolated from PBMC, cultured in RPMI, and stimulated with CpG (3 μ g/ml) in the presence or absence of rIL-37a and rIL-37b. Two doses (50 ng and 50 pg) of rIL-37a and rIL-37b were used. The IgG levels in the culture supernatants were measured by ELISA at day 3, 4 and 7. Data are mean \pm SD, * P < 0.05, ** P < 0.01, and *** P < 0.001 compared to control (non-stimulated or CPG stimulated cells). The figure represents three independent experiment.

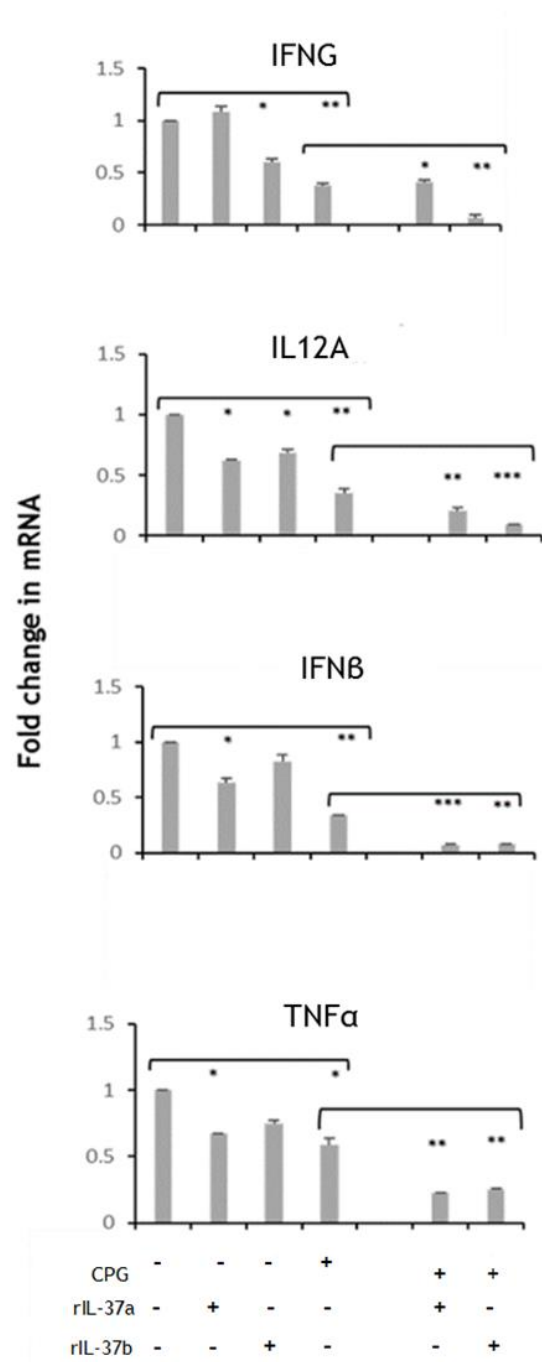


Figure 4.19 IL-37 regulates Th1 cytokine expression in human B cells stimulated with CpG.

Human CD19⁺ B cells were isolated from PBMC and stimulated with CpG (3 ug/ml) in the presence or absence of IL-37a and IL-37b (50 pg/ml). Cells were harvested after 3 day culture for TNF- α , IL-12 a, IFN β and IFN γ gene expression using qPCR. Data are mean \pm SD, *P< 0.05, **P< 0.01, and ***P< 0.001 compared to control (non-stimulated or CpG stimulated cells). The figure represents three independent experiment.

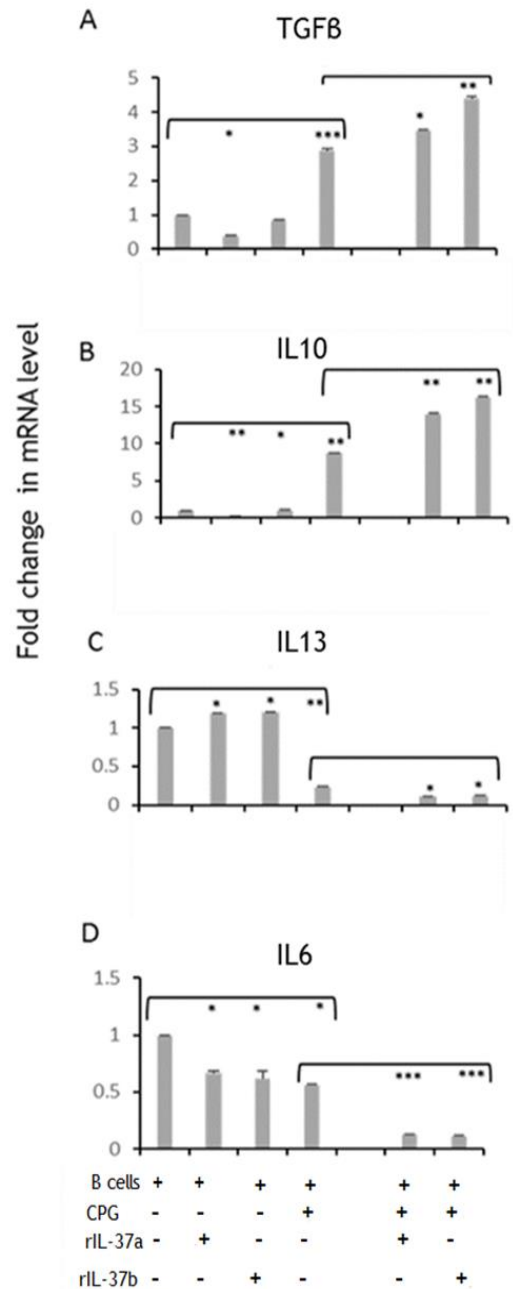


Figure 4.20 Effect of IL-37 on the regulation of T-reg and Th2 cytokine expressions in human B cells stimulated with CpG.

Human CD19⁺ B cells were isolated from PBMC and stimulated with CpG (3 ug/ml) in the presence or absence of IL-37a and IL-37b (50 pg/ml). Cells (10⁶/ml) were harvested after 3 day cultures for TGF- β , IL-10, IL-13 and IL-6 gene expression using qPCR. Data are mean \pm SD, *P < 0.05, **P < 0.01, and ***P < 0.001 compared to control. The figure represents three independent experiment.

4.2.4.2 Role of recombinant IL-37a and b in human B cell proliferation

As IL-37 can induce murine B cell proliferation, alone or in the presence of TLR signals, we investigated the effect of IL-37a and IL-37b on human B cell proliferation under different stimulus. B cell activation and proliferations require three signals from BCR, co-stimulation and cytokines (Nonoyama et al, 1993; Wortis et al, 1995; Dörner and Radbruch, 2007). Different cytokines may have different impact on B cell functions, including proliferation. IL2^{-/-} mice have an age-related decrease in B lymphocytes number (Schultz et al, 2001); while IL-21 is pivotal for normal plasma cell formation; as IL-21 deficiency affects affinity maturation production in germinal centre B cells (Linterman et al, 2010). We therefore compared the effect of the three signals, alone or in combination, on B cell proliferation. Here, we used anti-IgM, which cross-links the BCR to mimic the BCR activation, the co-stimulation signal of CD40L from T cells was provided from a fibroblast cell line 3T3 expressing transgenic CD40 ligand (Liebig et al, 2009). Cytokines, IL-2 and IL-21 that promote B cell proliferation and differentiation into plasma cells were also included in this study.

CD19⁺ B cells were isolated from human PBMCs, and cultured with different stimulators (IL-2, IL-21, CD40 and IgM), alone or in different combinations in the presence or absence of IL-37a or IL-37b for 3 days. B cell proliferation was measured by Brdu proliferation assay. I found that IL-37a and IL-37b alone slightly but significantly reduced human B cell proliferation (Fig 4.21). IL-2, anti-IgM antibody alone could enhance B cell proliferation, whereas IL-21 and CD40 alone had little effect on B cell proliferation in this experimental condition. However, the combination of two or three stimuli dramatically triggered B cell proliferation (Fig 4.21). I next determined the effect of IL-37a and IL-37b on the B cell proliferation induced by two or three of the activation signals as indicated in (Fig 4.22). The CD19⁺ B cells were isolated and cultured with different stimulators (IL-2, IL-21, CD40 and IgM), alone or in different combinations in the presence or absence of IL-37a or IL-37b for 3 and 5 days before the measurement of B cell proliferation. While IL-2 plus IL-21 failed to induce B cell proliferation, the presence of IL-37a but not IL-37b in this condition significantly enhanced B cell proliferation at day 3 but not day 5 culture. Anti-IgM and CD40 significantly induced cellular proliferation, however IL-37a and IL-37b failed to further

enhance the effect. Anti-IgM, CD40 and IL-21 induced significantly higher levels of proliferation in B cells, which, again, could not be further enhanced by IL-37a and IL-37b (Fig 4.22). In summary, in contrast to murine B cells, IL-37a and IL-37b have little effect on the human B cell proliferation. This is consistent with the failure of IL-37 in the induction of antibody production in human B cells. We also found that rIL-37a and rIL-37b had no influence on BCR, CD80/86 and CD40 expression on human B cells determined by flow cytometry (data not shown).

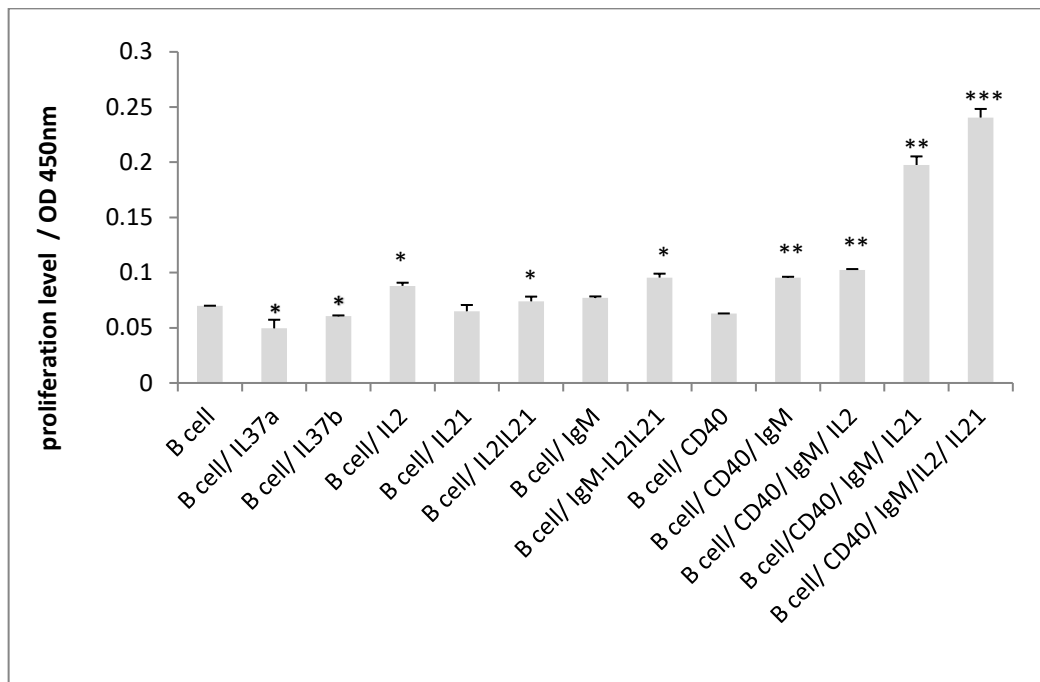


Figure 4.21 Set up and comparison the human CD19 B cells proliferation condition induced by different stimuli

Human CD19⁺ B cells were isolated from PBMC and cells (10^6 /ml), and cells were stimulated with anti-IgM (10 ug/ml), IL-2 (10 ng/ml), IL-21 (50 ng/ml), CD40 ligand (2 ug/ml), alone or in combination. In some samples, IL-37a and IL-37b (50 pg/ml) were also added into the B cell culture. Cells were harvested after 3 days cultures for proliferation assay using Brdu kit. Data are mean \pm SD, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control (B cells alone). The figure represents three independent experiment.

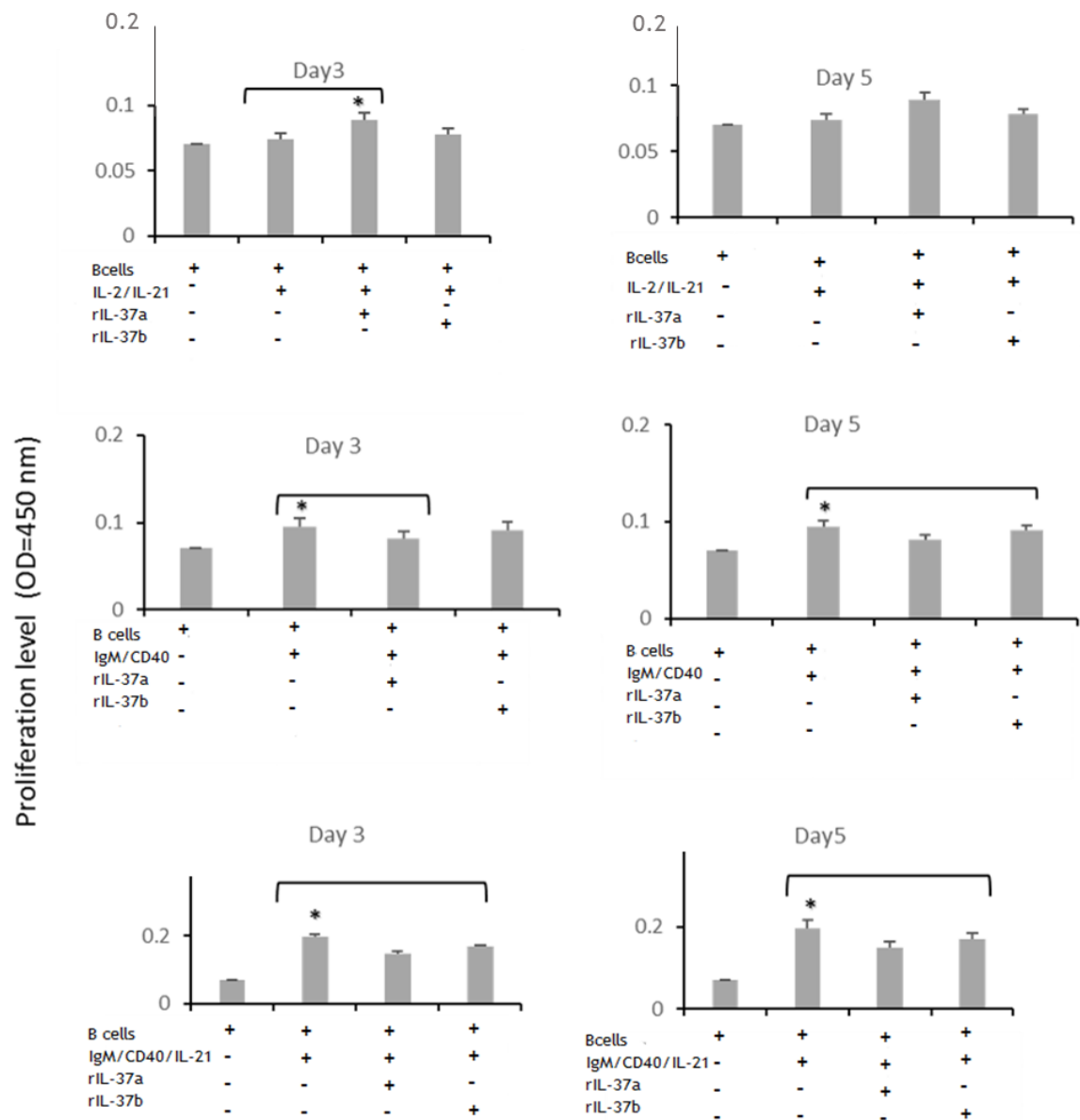


Figure 4.22 Role of IL-37a and IL-37b in the human B cells proliferation induced by different stimuli.

Human CD19⁺ B cells were isolated from PBMC and cells (10⁶/ml) were stimulated with (anti-IgM, IL-2, IL-21, CD40) in different combinations in the presence or absence of IL-37a and IL-37b (50 pg/ml). Cells were harvested after 3 and 5 days cultures for proliferation assay using Brdu kit. Data are mean \pm SD, *P < 0.05 and **P < 0.01 compared to control (IL-2, IL-21, anti-IgM/ CD40 or anti-IgM/ CD40/IL-21). The figure represents three independent experiment.

4.2.4.3 The expressions of IL-37 isoforms and receptors in human cells are regulated by immune activation signals and IL-37 it self

In order to reveal the mechanism underlying the failure of IL-37 in the induction of human B cell antibody production and proliferation, we next examined IL-37 isoforms and receptor expression and regulation in human B cells. Little is known how IL-37 isoform and receptor expressions are regulated in immune cells. We sought to study this in resting and activated B cells. CD19⁺ B cells were isolated from human PBMCs, cultured in medium only without treatment with stimulators. The RNA was extracted for the detection of IL-37 isoform expression by qPCR. We found that resting B cells did express all IL-37 isoforms (Fig 4.23). The cycle threshold (CT) value indicated that the relative expression level of IL-37a was the lowest and the IL-37b the highest among the isoforms in this context.

I next assessed if IL-37 can regulate its own expression in B cells. The B cells were cultured with or without rIL-37a or rIL-37b in culture for 10 days and the expression level of IL-37 isoforms detected by qPCR (Fig 4.24). Compared to the cell alone control, rIL-37a significantly downregulated all isoform expression, in particularly its own expression. In contrast, rIL-37b selectively suppressed IL-37a, IL-37c and IL-37e, but enhanced IL-37d expression. Intriguingly, rIL-37b has no effect on its own expression (Fig 4.24).

I further studied the regulation of IL-37 isoform expression in B cells activated with IL-2/IL-21 with or without rIL-37. Compared to the cell only control, B cell treatment with IL-2/IL-21 dramatically down regulated the expression of all IL-37 isoforms (Fig 4.25). IL-37a and IL-37b had minor effect on the IL-2/IL-21-mediated down regulation, with slightly enhanced IL-37b but reduced IL-37e expression in activated B cells (Fig 4.25). This was also the case in anti-IgM and CD40 signals activated B cells. Anti-IgM and CD40 treatment almost completely abolished all IL-37 isoform expressions and rIL-37 had little influence on this effect (Fig 4.26).

I next studied the regulation of IL-37 receptors, IL-1R8 and IL-18Ra expression in B cells. B cells cultured with or without rIL-37a, rIL-37b, IL-2/IL-21, anti-IgM/CD40 or CpG in the culture for 10 days and the expression level of IL-1R8

and IL-18Ra were detected by qPCR. As shown in Fig 4.25 and 4.26, rIL-37a slightly but significantly down regulated the expression of both receptors. On the other hand, rIL-37b reduced IL-1R8 but enhanced IL-18R expression. This was also the case in the CpG-activated B cells (Fig 4.26 and 4.27). The IL-2/IL-21 and anti-IgM/CD40 stimulation dramatically down regulated the expression of all the receptors (Fig 4.27 and 4.28). To support this finding, we found that IL-1R8 expression in B cells from wt and IL-37a-tg mice was also down regulated by LPS-stimulation (Fig 4.29).

To further support this finding, we also have evidence showing that activation signals and rIL-37 can also down regulate both IL-37 receptor expressions in human monocytes (Egle Katkeviciute, data not shown). Since IL-1R8 is a specific signalling receptor for IL-37 and can be down regulated by all the tested activation signals and IL-37 itself in B cells it is likely that all these signals can subsequently affect IL-37 signalling and function. This suggests that IL-37 expression and function are highly controlled in immune cells.

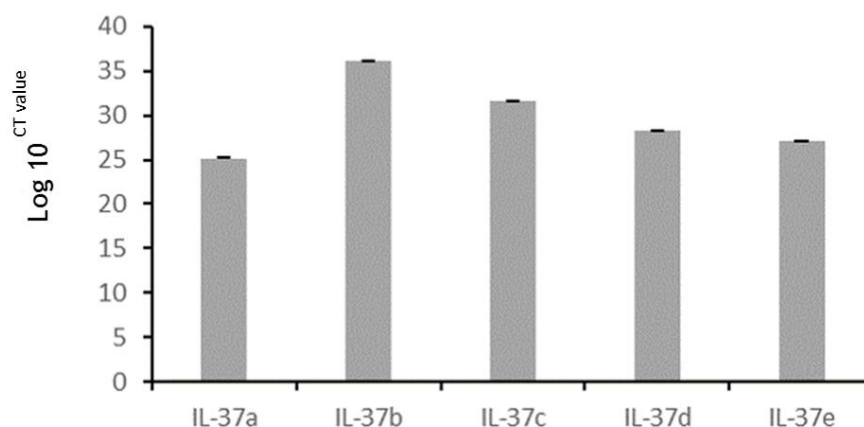


Figure 4.23 IL-37 isoforms expression in human CD19⁺ B cells

Isoforms at steady state; the graph shows all the five isoforms in CT value. Human CD19⁺ B cells were isolated from PBMC by MACS and cultured for long term 11 days, cells harvested and qPCR performed to measure IL-37 isoforms expression level. Human CD19⁺ cells express IL-37 isoforms at steady state; the graph shows all the five isoforms in CT value.

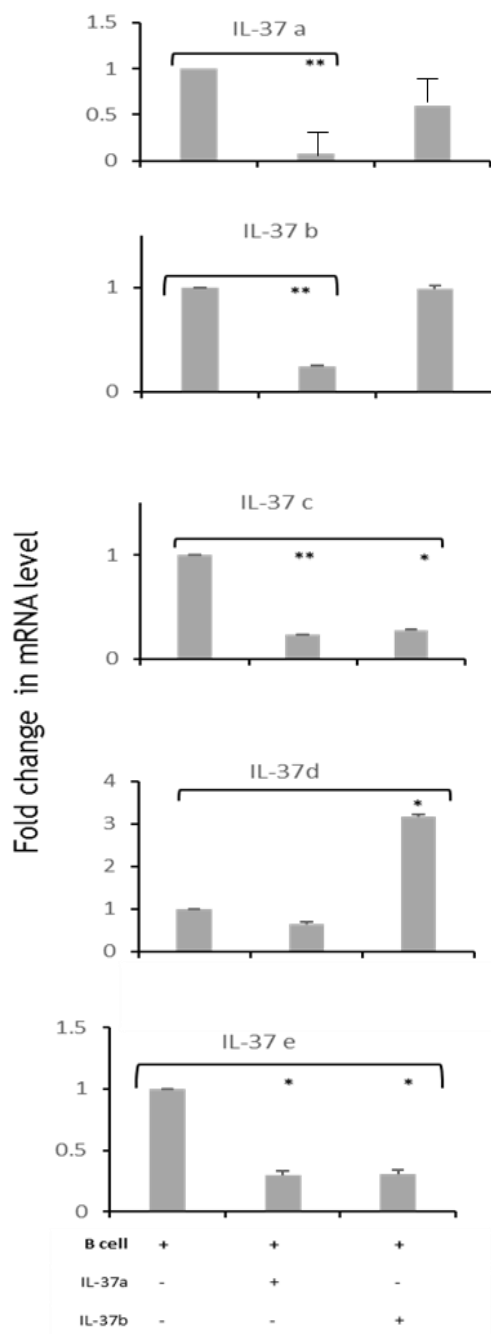


Figure 4.24 IL-37a and IL-37b regulation of IL-37 isoforms expression in human B cells.

Human CD19⁺ B cells (10⁶/ml) were isolated from PBMC and cultured with recombinant IL-37a and IL-37b (50 pg/ml) for 10 days. The cells were then harvested for IL-37 isoform expression by qPCR. Data are mean ± SD, *P < 0.05 and **P < 0.01 compared to control (B cells only). The figure represents three independent experiment.

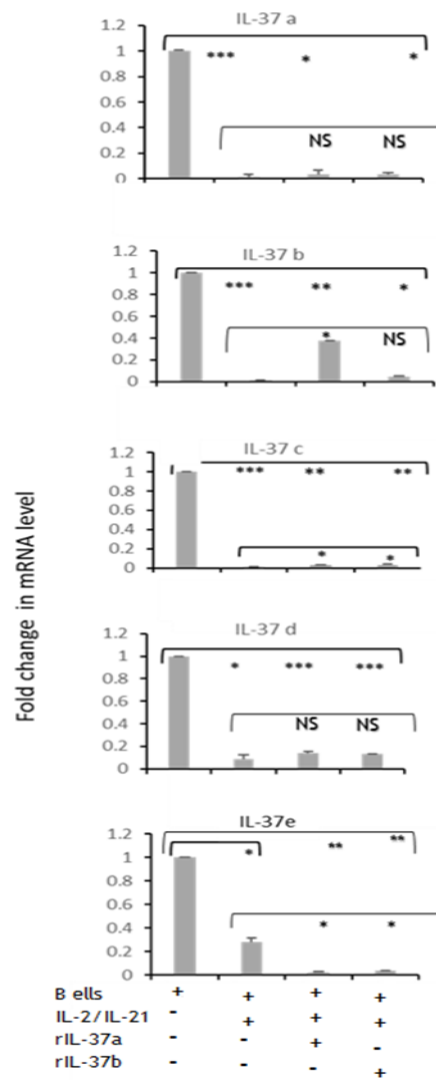


Figure 4.25 Regulation of IL-37 isoforms expression by IL-2/IL-21 in human B cell.

Human CD19⁺ B cells were stimulated with IL-2 (10 ng/ml) and IL-21 (50 ng/ml) followed by treatment with (50 pg/ml) of rIL-37a or b. After 10 days, cells were harvested for isoforms expression checking by qPCR. The figures shows that IL-2/IL-21 down regulate IL-37 isoforms. Using rIL-37a and rIL-37b with IL-2/IL-21 did not show any significant upregulation, except that, isoform b and c can be up regulated by IL-37a and IL-37b respectively. Data are mean \pm SD, NS represents (non-significant), *P< 0.05, **P< 0.01 and ***P< 0.001 compared to control (B cells only or B cells treated with IL-2/IL-21).

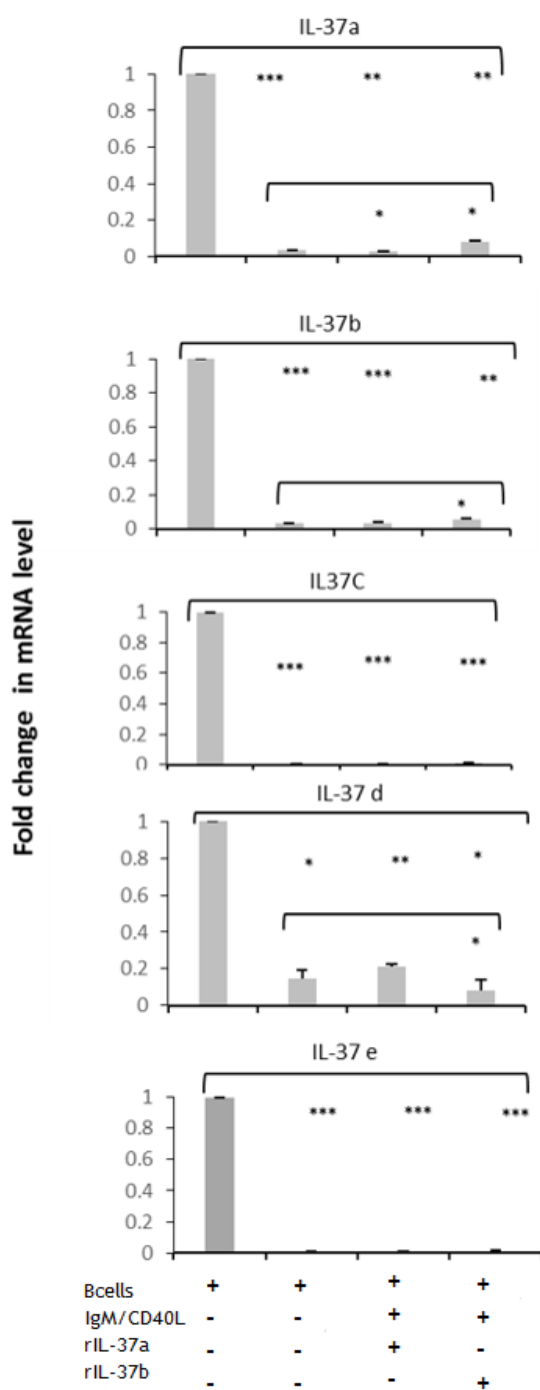


Figure 4.26 Regulation of IL-37 isoforms expression by IgM/CD40 in human B cells.

Human CD19⁺ B cells were stimulated with recombinant IgM/CD40L (10 ug/ml, 20 ng/ml) respectively, and IL-37 (50 pg/ml) for 10 days, the cells were harvested for IL-37 isoforms expression by qPCR. Data are mean \pm SD, *P < 0.05, **P < 0.01, and ***P < 0.001 compared to control (B cells only or treated with IgM/CD40). The figure represents three independent experiments.

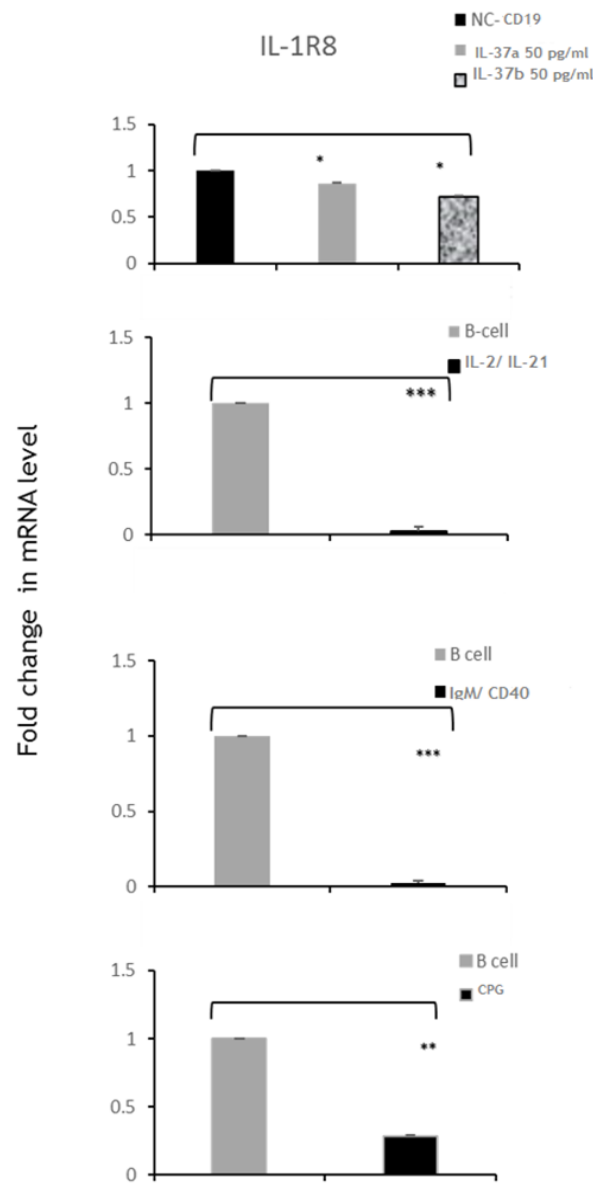


Figure 4.27 Regulation of IL-1R8 chain expression in human B cells.

Human CD19⁺ B cells were stimulated with or without rIL-37 (50 pg/ml), IL-2/IL-21 (10, 50 ng/ml) respectively, anti-IgM(10 ug/ml), CD40 (20ng/ml), CpG (3 ug/ml) for 10 days. The cells then were harvested and the expression level of IL-1R8 determined by qPCR. NC-CD19 represents B cells only or negative control. Data are mean \pm SD, *P< 0.05, **P< 0.01, and ***P< 0.001 compared to control. The figure represents three independent experiment.

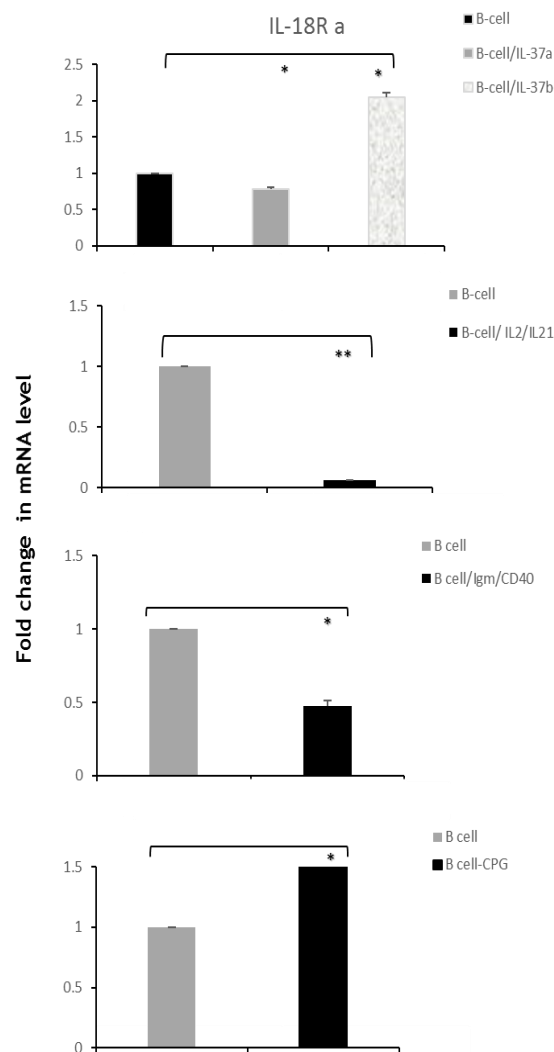


Figure 4.28 Regulation of IL-18Ra chain expression in human B cells.

Human CD19⁺ B cells were cultured with or without rIL-37, (50 pg/ml), IL-2/IL-21 (10, 50 ng/ml), anti-IgM/CD40 (10 ug/ml, 20 ng/ml), CpG (3 ug/ml) for 10 days. The cells then were harvested and the expression level of IL-18Ra determined by qPCR. Data are mean \pm SD, *P < 0.05 and **P < 0.01 compared to control (B cells only). The figure represents three independent experiments.

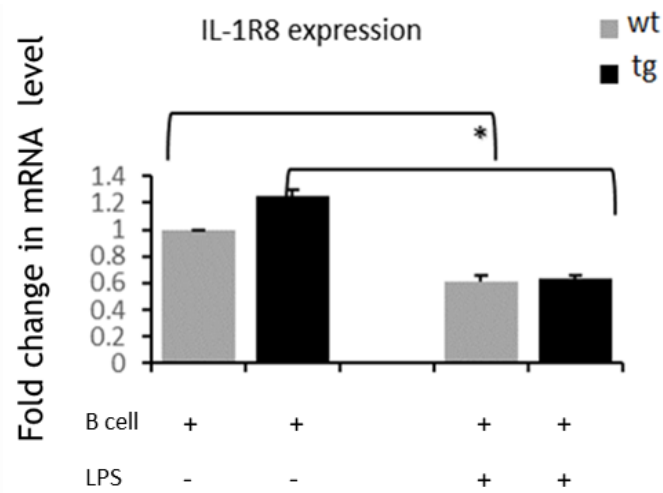


Figure 4.29 LPS regulates IL-1R8 expression in murine B cells.

CD19⁺ B cells were isolated from the spleen of wt and IL-37a-tg mice, and stimulated with LPS (100 ng/ml) for 3 days. The cells were then harvested for the detection of IL-1R8 expression by qPCR. Data are mean \pm SD, * P < 0.05 compared to control. The figure represents three independent experiment.

4.3 Discussion

As a first step toward the understanding of the function and importance of all IL-37 isoforms in immunity and disease, this study investigated the induction and bioactivity of IL-37a in human and mouse innate and adaptive immune cells. The preliminary results from this project demonstrated for the first time that: 1) the expressions of IL-37 isoforms are differently regulated in immune cells, depending on the activation conditions and also the dose and time of stimuli. IL-37a is one of the highly induced isoforms in human monocytes. 2) IL-37a is bioactive and a novel immunoregulatory cytokine. 3) While sharing functional similarity, IL-37a and IL-37b are different in the regulation of some cytokine productions. 4) IL-37 is not only immunosuppressive but may also be immunoactive, for instance in B-cell antibody production and proliferation. 5) The expression and function of IL-37 isoforms in immune cells are highly regulated by immune activation signals and by IL-37 itself. Thus, it is likely that IL-37a is an important IL-37 isoform and differs from IL-37b in the regulation of immune response in health and disease.

While the function of IL-37b as an important immunosuppressive cytokine has been well studied, the functions of IL-37a and other IL-37 isoforms are largely unknown. Using several *in vitro* experimental approaches, including IL-37a RNA knockdown, recombinant protein and transgenic cells, we demonstrated that IL-37a is bioactive and can regulate the expression of several TLR-induced inflammatory cytokines in macrophage, monocytes and B cells. Thus, IL-37a is a novel immunoregulatory cytokine in the IL-37 family and contributes to the IL-37-mediated immunoregulation and immunotolerance. However, the effect of IL-37a on health and disease is still unknown and currently under investigation in our laboratory using human IL-37a transgenic mice *in vivo*.

We also found that IL-37a and IL-37b share functional similarity in the tested experimental conditions; suppress the same cytokine expressions and with the similar effect. Whereas it is known that IL-37b triggers its anti-inflammatory signals through a receptor complex consisting of IL-18R α and IL-1R8 (Nold-Petry et al, 2015), how IL-37a signals is unknown. Given IL-37a and IL-37b share the common IL-1 like domain for receptor binding and signalling is likely that both

the isoforms signal via the same receptor. Our preliminary results demonstrated that anti-IL-1R8 blocking antibody or Sigirr^{-/-} mice could abolish the IL-37a-mediated effect in vitro, suggesting that rIL-37a also signals via the same receptor as IL-37b (D. Xu, unpublished result).

Intriguingly, FL-IL-37a is different from FL-IL-37b in the regulation of some cytokine gene expressions, including IL-37 (Fig 4.3, 4.6, 4.18 and 4.23). Furthermore, FL-IL-37a is more effective than FL-IL-37b in the suppression of LPS-induced IL-6 production in macrophages (Fig 4.4 and 4.5). The reason is still unclear and under investigation. Since IL-37a and IL-37b are completely different in their N-terminal sequences, which are encoded by exon 3 and exon 1 and 2, respectively, it is possible that the functional difference between the two isoforms may be attributed to their difference in N-terminal sequence. To support this, we found in Chapter 3, that IL-37a and IL-37b vary in their chemical features, including PI, molecular weight, and amino acids percentage, and IL-37a is less soluble compared to IL-37b. This may influence their receptor binding affinity and protein stability, thereby the bioactivity.

Another important difference between the two isoforms is that, IL-37a but not IL-37b possesses a NLS in the protein N-terminal. NLS is able to translocate proteins from cytoplasm to the cell nucleus where the protein can regulate gene expression (Jans and Hubner, 1996; Yoneda et al, 1997). It has been known that IL-1 α but not IL-1 β carries NLS and can regulate immune response in the cell nucleus (Luheshi et al, 2008). Further works needed to reveal whether IL-37a is a nuclear protein and whether it is capable of in the gene regulation in the nucleus. In Chapter 5, I analysed and identified the common and specific genes and signal pathways regulated by IL-37a and IL-37b in LPS-induced inflammatory response by RNA-sequence and bioinformatics. This will further enhance our understanding of the common and different between the two isoforms in immunoregulation and tolerance.

How the expressions of IL-37 isoforms are induced, and regulated in an immune cell population in a given condition is poorly understood. Since we have 5 IL-37 isoforms with possibly different functions it is important to know whether they are equally or differently induced and regulated in immune cells. Our results here demonstrated that IL-37 isoform expression is activation signal induced in

immune cells, including TLR signals. In addition, among the three isoforms (IL-37a, b, d) that possess the full IL-1-like domain and may have cytokine activity, IL-37a and IL-37b are highly induced compared to IL-37d isoforms. This suggests that IL-37a and b may be the dominant IL-37 isoforms with cytokine activity. We also noted that IL-37c and IL-37e are the most inducible IL-37 isoforms compared to others with IL-1-like domain. The reason is unclear, may be in part due to the IL-37e and c are shorter in gene and protein sequence compared to the others; and long or multi introns may cause delay in gene expression time. Since the two isoforms lack the full IL-1-like domain and are unlikely to have cytokine activity it has been suggested that IL-37c and e may interfere with other IL-37 cytokine expression by competing with RNA splicing and/or cleaving enzymes for maturation (Boraschi et al, 2011).

We also demonstrated that IL-37 isoform induction and regulation in human monocytes is dose and time-dependent; the lower dose LPS (100 ng/ml) and short activation time (within 16 hours) can upregulate the expression of IL-37 isoforms. In contrast, the higher dose LPS (200 ng/ml) and longer time stimulation may lead to the down regulation of IL-37 expression. This was also the case in the persistently activated human B cells. The reduced IL-37 expression in these conditions was not due to TLR-induced cell apoptosis and stress since the cells produced more inflammatory cytokines compared to the cells stimulated with lower LPS dose or shorter times (data not shown). The immunological and physiological meaning of the reversed association between immune cell activation and IL-37 expression is not yet fully understood. Given the main function of IL-37 is to suppress immune activation and inflammation; it is possible that the immune cell has to abolish IL-37 expression and function first in order to initiate an optimal immune activation and cellular proliferation.

How the strong activation signals down regulate IL-37 expression is unknown. Our preliminary results suggest that IL-37, in particularly IL-37a, can regulate the expression of all IL-37 isoforms. In addition, IL-37 and activation signals can also down regulate the IL-37 receptor expression in immune cells. Together, the two pathways will abolish IL-37 function by preventing the further production of endogenous IL-37 and shutting down exogenous IL-37 signalling. While the impact of the regulation on immune response is still largely unknown, these

results suggest that IL-37 function is carefully regulated in immune cells in order to achieve an appropriate level of immune response, for instance in B cell antibody production. B cells play an important role in immunity and in disease by presenting antigen, producing antibody and cytokines (Amlot et al, 1985; Rivera et al, 2001; Vazquez et al, 2015). Thus, B cell function must be tightly checked. The effect of IL-37b on innate immune cells and T cells has been studied and current evidence suggests that IL-37b is an immunosuppressive cytokine on these cells. However, its function in B cells has not been studied; I therefore studied the effect of IL-37a and IL-37b on mouse and human B cells. The most dramatic finding was that IL-37 is able to increase, not decrease, IgG production in murine, to a lesser extent, in human B cells.

The antibody enhancement effect of IL-37 was observed in several experimental conditions with both recombinant IL-37a and b and transgenic IL-37a in the LPS or CD40-stimulated B murine B cells, suggesting that it is a general function of IL-37.

The precise mechanism by which IL-37 promotes antibody production in B cells is still unknown. Since IL-37 can enhance B cell number in a proliferation assay, it is possible that IL-37 may promote antibody production by enhancing cell number. However, it is unclear at this stage whether the enhanced B cell number by IL-37 is really due to enhanced cellular proliferation or preventing B cell apoptosis. Cytokines play an important but different role in B cell proliferation and apoptosis (Itoh and Heroheta, 1995). Th1 and Th2 cytokines, IL-12, IFN γ and IL-13 have similar helping effect on B cell expansion and antibody production (Smith and Pottage et al, 2000). However, in our case, both Th1 and Th2 cytokines were down regulated by IL-37, suggesting that these cytokines are not responsible for the IL-37-induced B cell proliferation. Regulatory cytokine IL-10 can prevent B cell apoptosis and promote B cell antibody production via STAT3 (Itoh and Heroheta, 1995; Ding et al, 2016), and we found that IL-37 can enhance IL-10 production in both murine and human B cells. STAT3 deficiency in B cells promotes germinal centre (GC) B cell apoptosis (Ding and Chen et al, 2016). It has been reported that IL-37b upregulates STAT3 in IL-1R8 dependent pathway, which is critical for IL-10 signal (Nold-Petry et al, 2015); therefore, it is possible that IL-37 may promote B cell proliferation and IgG production by

inducing IL-10 production, which keeps B cell survival and differentiation. However, IL-10 was only enhanced one day, but not after that, by LPS stimulation in IL-37 transgenic B cells. TGF- β can also be enhanced by IL-37 in murine and human B cells. GC B cells differentiate into memory-like B cells and plasma cells dependent on endogenously secreted IL-10 and TGF- β (Durand et al, 1993). However, TGF- β can also suppress mature B cell proliferation (Tsuchida et al, 2017). Its role in IL-37-induced B cell proliferation and antibody production is unclear.

We also assessed the expression of key B cell signalling molecules in IL-37-mediated B cell proliferation and antibody productions, Lyn, SHIP-1 and April (Muta et al, 1994; Brauweiler et al, 2000). Our results showed that IL-37 signals increased the expression of Lyn but decreased SHIP-1 and had no significant effect on April. SHIP-1 plays a regulatory role in B cell signaling. *Inpp5d* $-/-$ mice exhibit enhanced survival and are hyperresponsive to BCR stimulation, and elevated serum Ig (2, 3, and 4). Studies have showed that SHIP-1 promotes NF- κ B dependent cytokine and MAP kinase activation exploiting TLR4-independent PI3K/Akt pathway; therefore, decrease in SHIP-1 was accompanied with increased survival and reduced inflammatory response (Brauweiler et al, 2000; Li et al, 2016). Thus, down regulation of SHIP may contribute to IL-37-enhanced antibody production and proliferation in B cells. However, the underlying mechanism by which IL-37 downregulates SHIP in B cells is currently unknown.

Current evidence suggests that Lyn is a dual functional enzyme, which can positively and negatively regulate B cell function (DeFranco et al, 1998). Lyn depletion in B cells results in a lupus-like autoimmune disease (Lamagna et al, 2013; Li and Fang et al, 2016). Its role and down regulation in IL-37-mediated B cell activation need to be further studied.

Because the marked effect of IL-37 on IgG production in murine B cells we further studied the effect of IL-37 on human B cells. I found that, different from in murine B cells, rIL-37 only had minor effect on human B cell antibody production. The effect was only seen in earlier (day 4), but not later (day 7 and 9) cultured B cells and only induced by lower but not higher dose of rIL-37 (Fig 4.17). Inconsistently, the minor effect of IL-37 on antibody production was

accompanied by minor or no effect for IL-37 on the B cell proliferation induced by different stimulus (Fig 4.17). The reason for the contrary effect of IL-37 on antibody production in human and murine B cells is understood. As IL-37 induced similar cytokine profiles in human as murine B cells, reduced Th1 and Th2 cytokines but enhanced regulatory cytokine IL-10 and TGF- β , thus, it is unlikely that cytokine profiles are responsible for the controversy. Because the main difference between human and murine B cells in this cellular context is that human but not murine cells express IL-37 isoforms, we sought to see if it is due to the dysregulation of endogenous IL-37 and receptors. We found that rIL-37a can down regulate the expression of all IL-37 isoforms, including its own expression, in the cultured B cells. In addition, rIL-37 and activation signals can also down regulate the IL-37 receptor IL-1R8 expression in both murine and human cells. Together, the two pathways may abolish IL-37 signalling and function in B cells, including antibody production. While this may provide a possible explanation for the low efficiency of IL-37 on human B cell antibody production, it can still not explain why IL-37-mediated effect on cytokine production in human B cells was not affected. This may be because the IL-37 and receptor induction is time-dependent enhanced first, then down regulated and the induction of cytokine expression is much quicker (within 24 hours) than antibody production (3-7 days). More detailed kinetic study of the induction of IL-37 and receptor expression may help to answer the question, another question is, if this is the case, why cannot the IL-37a and receptor expression be regulated in mouse B cells? This may be due to the follow reasons: 1) since mice do not have IL-37 gene it is possible that mice may also lack the regulatory system for the control of IL-37 expression; 2) it is also possible that IL-37 receptor system is less regulated in mouse compared to that in human cells.

Nevertheless, our results suggest that IL-37 has potential to promote B cell antibody production. Since overproducing antibody, in particular autoantibody is dangerous to the host, the production and function of IL-37/receptor system must be carefully controlled. Thus, it is worthwhile to study in detail how IL-37/receptor system is regulated in human B cells and whether the dysregulation of IL-37/ receptor expression may lead to autoimmune disease, such as SLE.

Chapter5

**Determine the difference between
IL-37a and IL-37b in the regulation of
transcriptome**

5 Determine the difference between IL-37a and IL-37b in the regulation of transcriptome

5.1 Introduction

The studies in Chapter 4 have demonstrated that IL-37a is bioactive and is a novel immune regulator. It suppresses LPS-stimulated inflammatory cytokine expression in monocytes and macrophages. In contrast, it also enhanced LPS-induced IgG production in B cells. In addition, IL-37a and IL-37b exhibit similarity and difference in biology and function. However, the molecular explanation for their similarity and difference in bioactivity is still lacking. In this chapter, using LPS/TLR4-induced inflammatory response in spleenocytes as a cellular model, I performed transcriptomic experiment to reveal the common and unique aspects of IL-37a and IL-37b in signalling and gene regulation of the LPS response. This part of the work was aimed to reveal some molecular explanations for their regulatory role in the LPS/TLR4 signalling-mediated effect on macrophages and B cell functions described in Chapter 4.

TLR4 signalling in innate immune cells including macrophages has been studied, and described in detail in (Chapter 1). Briefly (Fig 5-10), TLR4 signals via a MYD88-dependent and TRIF-dependent pathway. The former is triggered by an agonist binding to the cell surface TLR4 complex, and the recruitment of MYD88 and adaptor TIRAP (MAL), and the activation of signalling cascades nuclear factor kappa-light-chain (NF- κ B), and mitogen-activated protein (MAP) kinases; resulting in inflammatory gene expression. The latter is elicited when the TLR4 is internalised into cell plasma and the recruitment of TRAM and adaptor TRIF leads to the activation of IFN response factors (IRFs) and subsequent type I IFN expression.

BCR signalling is triggered by the antigen-BCR interaction; three important protein tyrosine kinases (PTKs) are then activated, the Src-family kinase SYK, Lyn and the TEC-family kinase BTK (Fig 5-11). The deletion of either SYK or Lyn is sufficient to largely abolish BCR signalling (Gross et al, 2011; Ackermann et al, 2015). Lyn is the predominant Src-family tyrosine kinase in B cells. Current evidence suggests that Lyn is a dual functional enzyme, which can positively and

negatively regulate B cell function (Nishizumi et al, 1998). Lyn deletion in B cells results in enhanced cell proliferation and lupus-like autoimmune disease (Li and Fang et al, 2016). These tyrosine kinases will then activate downstream BCR signalling effectors, including the important PI3K and phospholipase C-gamma 2 (PLC- γ 2) and B-cell linker protein (BLNK). This signalling ultimately results in the activation of calcium signalling, MAPK and NF- κ B pathways and the expression of genes involved in B cell proliferation, differentiation, cytokine and Ig production as well as other functions (Fig 5-11) (Nishizumi et al, 1998; Li and Fang et al, 2016). BCR signalling is tightly regulated, primarily by SHP-1 and SHIP (O'Neill et al, 2011; Getahun et al, 2016). Both inhibitors share regulatory function in BCR signalling, but via different acting mechanisms (O'Neill et al, 2011). SHP-1 is a protein tyrosine phosphatase that counters BCR signalling at early stages by dephosphorylating activating signalling molecules, such as Spleen tyrosine kinase (SYK), and BLNK (Adachi et al, 2001). SHP-1 deficiency results in increased serum levels of IgM, IgG1, and IgG3 (Alsadeq et al, 2014). Furthermore, in mice, B cell-specific loss of SHP-1 leads to an accumulation of B-1a cells and systemic autoimmunity (Pao et al, 2007). SHIP is a lipid phosphatase that removes the signalling lipid phosphatidylinositol 3, 4, 5-trisphosphate (PIP3), and thereby impairs BCR signalling pathway (Baracho et al, 2011). SHIP-1 deficiency mouse had elevated IgG serum Abs, and developed lupus-like phenotypes (O'Neill et al, 2011; Chen et al, 2017). Thus, SHP-1 and SHIP are required for B cell tolerance and the prevention of the development of B-cell mediated autoimmunity (Getahun et al, 2016).

While TLR ligands have a profound effect on B cell proliferation, differentiation and antibody production, the precise mechanism and signalling pathways are still not known. Recent evidence suggests that TLR4 signals in B cells through two distinct pathways, one via the BCR leading to activation of SYK, ERK, and AKT and this pathway is independent of MYD88 (Schweighoffer et al, 2017). In addition, the MYD88-independent pathway leads to the B cell proliferation and activation (Yanagibashi et al, 2015). The other pathway through MYD88 leads to activation of NF- κ B (Bekeredjian-Ding and Jegou, 2009; Schweighoffer et al, 2017). However, the function of the MYD88-dependent pathway in this context has not been investigated. In another report, it has been suggested that MYD88 and TRIF pathways regulate B cell response differently;

immunization induced IgG1 production was impaired in *Ticam2*^{-/-} mice, while the antibody was enhanced in *Myd88*^{-/-} mice (Pihlgren et al, 2012; Yanagibashi et al, 2015). Furthermore, the T-cells ID antigen IgG response was strictly dependent on TLR4 and BCR signals and the IgG class switch was mediated by TRIF, but not by MyD88 (Pihlgren et al, 2012). However, whether IL-37 regulates BCR and TLR signalling in B cells is unknown, and the understanding of IL-37b signalling may help to understand IL-37a signalling in immunoregulation.

Current evidence suggests that IL-37b signals with the receptor-dependent and independent pathways in gene regulation (Chapter 1). In brief, the extracellular IL-37b can bind to and signal via the receptor complex consisting of IL-18R α and IL-1R8 (Nold-Petry et al, 2015). The same as other members in the IL-1 family, the IL-37 receptor-mediated signalling pathway is also MYD88 dependent (Nold-Petry et al, 2015). The receptor-independent pathway is less understood. It takes place intracellularly and may be via Smad3 and TGF signalling pathway (Luo et al, 2017). While the study of IL-37 signalling transduction has just started, one report has revealed the difference between the two pathways in IL-37b-mediated gene regulation (Nold-Petry et al, 2015). The spleenocytes RNA was isolated from wild type, IL-37b-tg and IL-37b-tg/*sigirr*^{-/-} mice, 3 hours after intraperitoneal injection of LPS, and the immune gene profiles were analysed by RNA-sequencing along with the Innate Immune Database (IDB). About 2,000 genes associated with innate immune response have been identified and the genes regulated by receptor-dependent and independent pathway of IL-37b were further identified by comparing the gene profiles among the wt, IL-37tg and IL-37b-tg/*sigirr*^{-/-} mice groups. The results indicate that: 1) IL-37b not only suppresses, but also enhanced gene expressions compared to the wt control with the suppression effect dominant; 2) in the receptor-dependent manner, IL-37b primarily inhibited genes that are largely associated with inflammatory response, including inflammatory cytokines, mediators and signalling molecules. In addition, the receptor-dependent manner, IL-37b induced some genes with immunoregulatory property; but also some inflammatory genes; 3) in the receptor-independent fashion, IL-37 inhibited and enhanced the expression of many genes including the genes involved in inflammation, metabolism, signalling transduction and chemokines; 4) IL-37b seems to have a wide ranges of effects

on the regulation of genes in different pathways. The wide regulatory effects of IL-37b have been further confirmed totally and by phosphorylated protein arrays with isolated macrophages and DCs from the spleens of wt, IL-37tg and IL-37b-tg/sigirr^{-/-} mice (Nold-Petry et al, 2015). This work has established IL-37b signalling and function in gene regulation. However, how IL-37a signals in gene regulation is unknown and the research in IL-37b signalling is helpful for the understanding of IL-37a signalling and the difference between the two isoforms. Based on all the results from our own and from other labs, I propose that: the functional similarity and difference between IL-37a and IL-37b can be attributed to their difference in protein sequence and signalling transduction. Using their shared C-terminal protein sequence containing IL-1-like domain for receptor binding and signalling, IL-37a and IL-37b may regulate common signalling pathways and genes. However, given their difference in N-terminal sequence, they may regulate different pathways and genes. The overall aim in this chapter was to test this hypothesis using LPS/TLR4 activated murine splenocytes to understand how IL-37a and IL-37b regulate TLR signalling transduction and what is common and different between the two IL-37 isoforms in gene regulation.

The aims of the project are:

1. Identify the different expressed genes regulated by IL-37a and IL-37b with microarray and bioinformatics assay
2. Assess the immune genes/signalling pathways, which are commonly regulated by both IL-37a and IL-37b.
3. Identify the immune gene/pathways specifically regulated by IL-37a or IL-37b.
4. Reveal the possible molecular mechanism by which IL-37a and IL-37b regulate LPS/TLR4-mediated inflammatory response from the transcriptomic.

5.2 Results

5.2.1 Identifying the LPS-induced genes differentially regulated by IL-37a and IL-37b

To understand the common and variable effect of IL-37a and IL-37b in gene regulation, we next systematically identified and compared genes regulated by IL-37a and IL-37b using transcriptomic analysis of RNA. Briefly, spleenocytes from wt, IL-37a-tg and IL-37b-tg mice (n=3) were stimulated with LPS (500 ng/ml) for 4 hours. Total RNAs from each mouse were isolated and cDNA synthesised by reverse-transcription. The gene profiles were performed using microarray (Chapter 2), and analysed using the Database for Annotation Visualization and Integrated Discovery (DAVID)/bioinformatics (<http://david.abcc.ncifcrf.gov>).

In order to identify the differentially expressed genes between IL-37a and IL-37b, we initially identified global differentially expressed genes (DEG) between LPS-stimulated wt and IL-37a-tg or IL-37b-tg spleenocytes. Firstly, the global DEG between IL-37a-tg and wt control were identified (Fig 5.1). Euler diagrams show (A) the down and (B) upregulated genes by IL-37a compared to wt control cells. Overall, 2905 genes were significantly down regulated by IL-37a (Fig 5.1a). Unexpectedly, 2881 genes were also upregulated by IL-37a versus the wt control (Fig 5.1b). By using the same strategy, the global DEG between IL-37b-tg and wt control were also identified (Fig 5.2). Compared to the control, 5263 genes were down regulated by IL-37b (Fig 5.2A). Compared with the control, 4666 genes were also upregulated by IL-37b (Fig 5.2B).

Next, the common and specific genes between IL-37a and IL-37b groups were identified by comparing the IL-37a and IL-37b specific DEG identified above. As illustrated in the (Fig 5.3A), among the downregulated DEG between the IL-37a and IL-37b gene pools, 1723 DEG were the common genes that were down regulated by both IL-37a and IL-37b (27%, common genes/total genes). 1182 genes were found to be specifically down regulated by IL-37a and 3540 genes were specifically down regulated by IL-37b (Fig 5.3A).

By comparing the upregulated DEG of IL-37a and IL-37b identified above, we found that 1356 genes (about 22%) were shared by the two IL37 isoforms (Fig 5.3b). 1525 genes were specifically upregulated by IL-37a and 3310 by IL-37b.

Furthermore, it was noted that IL-37b regulated more genes than IL-37a in the experimental conditions; with 2.17 folds more in the down regulated genes and 2.99 folds more in the upregulated genes. Thus, IL-37a and IL-37b are largely different in gene profiling, while sharing some gene profiles. This is consistent with the results in Chapter 3 and Chapter 4, that IL-37a and IL-37b share C-terminal protein sequence but are different in N-terminal sequence and display functional similarity and verity in biology.

5.2.2 Revealing the common signalling pathways/genes regulated by both IL-37a and IL-37b

In order to understand the molecular mechanism by which IL-37a and IL-37b regulated innate and adaptive immune cell function performed in Chapter 4, I next assess the gene function by associating the IL-37a and IL-37b regulated immune genes with signalling pathways. IDB has been selected for this purpose, which was generated from several major databases and focused on, but not limited to the innate immunity by curating experimentally validated human and mouse interactions from the biomedical literature (Lynn et al, 2010). It provides a manually curated knowledge base of the genes, proteins, and particularly, the interactions and signalling pathways involved in mammalian immune response, including TLR-mediated response (Lynn et al, 2008; 2010; Breuer et al, 2012). Since TLR, cytokines and B-cells were involved in Chapter 4, the TLR signalling and related cytokine/chemokine, JAK-STAT, NOD and B cell pathways were further studied in detail. Firstly, gene ontology of these common regulated genes by both IL-37a and IL-37b identified in (Fig 5.3) was assessed using IDB, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The results revealed that IL-37a and IL-37b significantly repressed genes associated with 20 signalling pathways and the number one pathway suppressed by both the cytokines was the TLR signalling pathway (Fig 5.4A). Both the cytokines also significantly upregulated genes in 10 pathways (Fig 5.4B). The top enhanced pathway was the cytokine-cytokine receptor interaction. The key and related signalling pathways/genes were studied in more detail (Table 5.1A and B).

Agreeing with my previous results in Chapter 4, showing that IL-37a and IL-37b inhibited TLR4-induced inflammatory cytokine production in macrophages, here both the cytokines also suppressed several signalling components in the TLR pathway (Table 5.1A and fig 5.5A).

These included important TLR4 co-receptor CD14 and MyD88 adaptor protein TIRAP, that play an initial role in TLR signalling (Zanoni et al, 2011), and the downstream signalling molecules MAPK3 and 14 that encode p38 α , NF- κ B elements and Pik3cd (Table 5.1A). The Nod1 in Nod-like receptor (NLR) pathway were also inhibited, which is required for the inflammasome formation and IL-1 β and IL-18 maturation and secretion (Kavathas et al, 2012; Moreira and Zamboni, 2012). Agreeing with the TLR signalling inhibition and our results in Chapter 4, IL-37a inhibited a number of inflammatory cytokine/receptor genes, including *Il1b*, *Il18*, *Il17a*, *Ifnar1* (encoding Ifn alpha/beta receptor alpha chain), *Kdr* (encoding VEGF receptor), *Tnfrsf1a* (encoding Tnfa receptor 1), *Ltbr* (encoding lymphotoxin B receptor) and several chemokines/receptors. Furthermore, IL-37a impaired the expression of key genes in MAPK signal pathway, including *Map3k5*; *MAP3k7*; *Mapk14*; *Mapk3* and *Pla2g4a* encoding cytosolic phospholipase A2 for the metabolic production of leukotrienes and prostaglandins (Zhu et al, 2001). Somegs in BCR signalling pathway, *Mapks*, *Rac1*, *Nfkb1*, *Pla2g4a* and *Plcg2* were also inhibited. The *Ptpn6* gene encodes the Src homology region 2 domain-containing phosphatase-1 (SHP-1), genetically loss of SHP1 leads to the development of B lymphopoiesis and systemic autoimmunity (Cyster and Goodnow, 1995; Pao et al, 2007). Interestingly, *Ptpn6* was also suppressed by both IL-37 isoforms. This may provide some explanation for the IL-37-enhanced B cell antibody production in mice (Chapter 4, fig 4.10 and fig 4.11).

IL-37a also enhanced the expression of some immune genes in associated pathways (Table 5.1B and fig 5.5B). Both isoforms also elevated the expression of some cytokines/chemokines/receptors and related JAK -STAT pathway: *Il4ra* encoding IL-4 receptor and IL-4 protects from LPS shock in mice (Grohmann et al, 2000); *Il12a* encoding IL-12a chain may have regulatory effect on IL-12 function (Presky et al, 1998). However, while the impact is still unknown, some inflammatory cytokine and chemokine receptor expressions were also enhanced by IL-37, including IL-19, an inflammatory cytokine, IL-6st encoding IL-6 Signal

transducer and 3CXC chemokine receptors. The enhanced expression of cytokines/chemokines was also accompanied by the increased expression of STAT3 and STAT6 in JAK-STAT pathway. Whereas MYD88 adaptor protein TIRAP was inhibited, both isoforms enhanced expressions of Ticam1 encoding TRIF and also Tlr6 and Tlr7 signalling with TRIF pathway, suggesting that both isoforms may promote TRIF-dependent TLR pathway (Nold-Petry et al, 2015). It was noted that MYD88 expression was also enhanced by IL-37, while MYD88 adaptor TIRAP was reduced by the two isoforms as described above; the biological meaning is still unclear.

5.2.3 Identifying the signalling pathways/genes specific regulated by IL-37a only

Next, the IL-37a specifically regulated genes identified in (Fig 5-3) were further analysed for their association with signalling pathways as above. While IL-37a specifically inhibited 1182 but enhanced 1525 genes compared with IL-37b (fig 5.3A and B), it inhibited more signalling pathways than it enhanced (Fig 5.6A, B). As shown in (Fig 5-6), IL-37a significantly down regulated more than 20, but only enhanced 10 pathways. The most significantly inhibited pathway was TLR (Fig 5-6A and table 5.2A). The most enhanced pathway was PPAR γ (Fig 5.6B and table 5.2B). The related key pathways/genes were further analysed (Table 5.2A and Fig 5-7A). IL-37a specifically down regulated TLR1 and 2 expressions in TLR pathway, suggesting that IL-37a can also suppress other TLR functions, beyond TLR4 and TLR9. Intriguingly, while it enhanced TRIF and TLR6 and TLR7 expression, it suppressed the expression of downstream TLR signalling pathway elements *Irf7* (Interferon regulatory factor 7), a transcription factor of the INF regulatory factor family which is involved in the type I INF production in both the MYD88 and TRIF-dependent TLR pathways and also *Ifnb1* encoding IFN β 1 (Uematsu and Akira, 2007). Furthermore, the *Ifnb1* down regulation was also accompanied by the down regulation of Jak2 in *Cblc* in JAK-STAT pathways.

IL-37a also specifically inhibited cytokine Il15 and Il1r2 (IL-1 receptor) in cytokine-cytokine receptor interaction pathway. 2 genes in BCR signalling pathway; glycogen synthase kinase 3(*Gsk3b*), a metabolic sensor that promotes the survival of naive recirculating B cells by restricting cell mass accumulation, and Phosphoinositide-3-kinase adaptor protein1 (*Pik3ap1*), a

signalling adapter linking BCR signalling to the PI3K-Akt signalling pathway. The genes/pathways specifically enhanced by IL-37a were also identified (Fig 5.6B, table 5.2B and fig 5.7b). IL-37a increased expression of several immunoregulators with known anti-inflammatory activity in TLR4 signalling, such as *Pltp* in peroxisome proliferator-activated receptors (PPAR γ) pathway, which is a LPS binding protein and blocks LPS function (Hailman et al, 1996; Gautier et al, 2008). PPAR γ , which inhibits TLR4-mediated inflammation (Appel et al, 2005), *Fzd1* encoding Frizzled-1, which induces Wnt/beta-catenin signalling to promote anti-inflammatory function in macrophages (Neumann et al, 2010). IL-37a also enhanced expression of the type II cytokine IL-13 which protects mice from LPS-induced lethal endotoxemia (Muchamuel et al, 1997). Altogether, these effects may ameliorate LPS and TLR-mediated inflammatory response thereafter-enhancing LPS tolerance and survival.

5.2.4 Identifying the specific signalling pathways/ genes regulated by IL-37b

The IL-37b specifically down regulated (3540) and upregulated (3310) genes (Fig 5-3) were analysed for the signalling pathways as above. The result showed that IL-37b significantly inhibited but also enhanced more than 20 signalling pathways (Fig 5.8A, and B). The most significantly down regulated pathway by IL-37b was the cytosolic DNA-sensing pathway (CDSP) which is used by cytoplasm PRRs to detect foreign double-strain DNA from microbes and trigger innate immune responses to produce type I INF and inflammatory cytokines. The most upregulated pathway was TLR pathway (Fig 5.8 A and B). Intriguingly, its signalling enhancement effect was greater than its inhibitory effect ($\log_{10} p\text{-value} = 10$ vs $\log_{10} p\text{-value} = 4$) (Fig 5.8A and B). The detailed analysis (Table 5.3A and Fig 5.9A) revealed that IL-37b selectively suppressed 4 genes in TLR pathway, including the important transcription factors *Akt1* and *Irf3*. The expression of 5 genes in the CDSP and 3 genes in NOD-like receptor (NLR) signalling pathway was also inhibited by IL-37b in the LPS-stimulated cells. While suggesting that IL-37b may also be able to regulate other pathogen pattern recognition pathways, its impact on LPS/TLR4 signalling needs to be evaluated.

IL-37b also specifically suppressed 3 important genes in BCR pathway, *Akt1*, *Lyn* and *Inp5d* encoding SHIP, a negative regulator of BCR. The down regulation of

SHIP by IL-37 is consistent with the result in B cells in Chapter 4. IL-37b also repressed 4 different genes in JAK-STAT pathway and related cytokine IFN γ , IL-12 α , and also IL-1R1, *Tnfsf9* encoding 4-1BBL (4-1BB ligand), a co-stimulatory molecule. *Socs1* is an important cytokine regulator, but also suppressed by IL-37b.

The genes/pathways specifically upregulated by IL-37b are shown in (Table 5.3B) and (Fig 5-9B). In TLR pathway, IL-37b enhanced expression of 10 genes including *Tlr4*, *Irak4*, *3Mapk* members and *pik3cg*. In cytokine/receptor interaction pathway, IL-37b increased transcription of cytokine/chemokine genes, including *Csf2*, *Il21*, *Il2rb*, *Tnf*, and *Vegfc* encoding Vascular endothelial growth factor C (VEGF-C); *Tnfsf11* encoding receptor activator of nuclear factor kappa-B ligand (RANKL); and Chemokine (C-X-C motif) ligand 2 (*Cxcl2*). The *Csf2*, *Il21* and *Il2rb* are associated with the JAK-STAT pathway. In BCR pathway, the *Mapk1* and Neuroblastoma *Ras* viral oncogene homologue (*Nras*), a member of Ras family in MAPK pathway, and *Nfatc2* in calcium signalling pathway were also down regulated (Table 5.3B and fig5.9B).

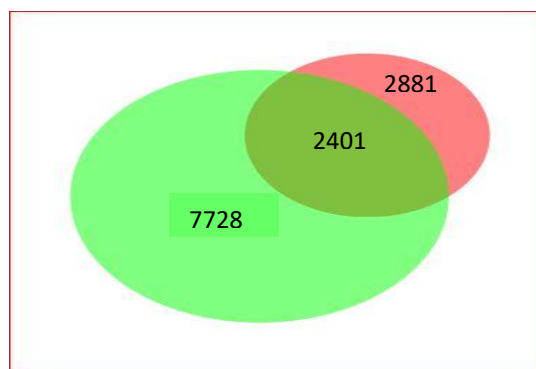
A: wt > IL-37a-tg**B: wt < IL-37a-tg**

Figure 5.1 Identification of the differentially expressed genes regulated by IL-37a in LPS stimulated splenocytes

Splenocyte cells from wt and IL-37a-tg mice were stimulated with LPS for 4 hours; total RNAs from each group were isolated for microarray. The differentially expressed genes identified using bio Venn programme and visualised by the area-proportional and overlapping using bioven Euler graphs. A) shows differentially expressed genes (DEGs) that are specifically down regulated by IL-37a (the red area) compared with wt control; while the green area are genes that are not affected by IL-37a. B) The red area shows DEGs that are specifically upregulated by IL-37a; the overlap area are the common genes between IL-37a and wt; while the green are genes that are not affected by IL-37a.

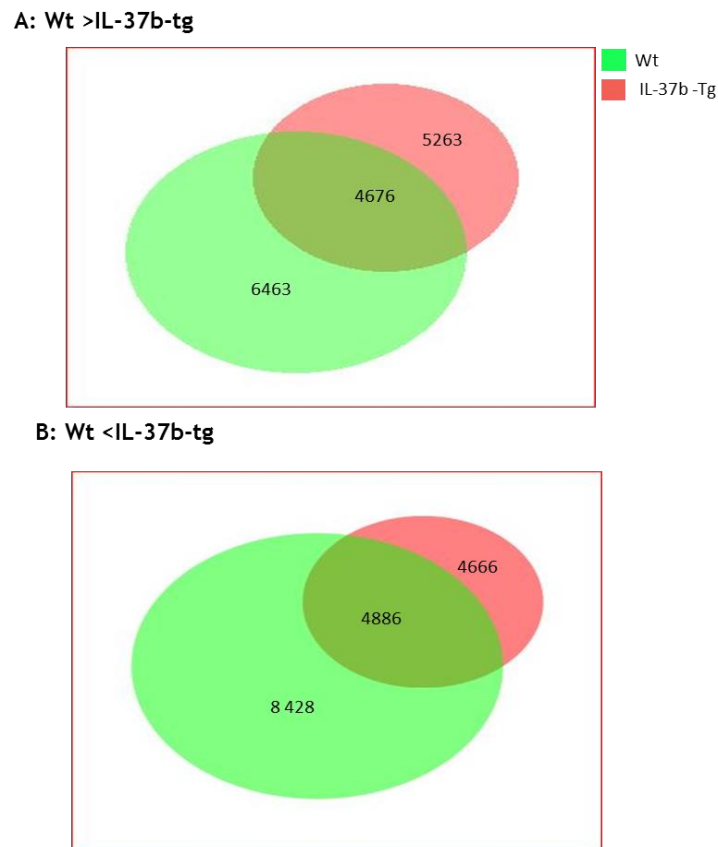


Figure 5.2 Identification of the differentially expressed genes regulated by IL-37b in LPS stimulated spleenocytes.

Spleenocyte cells from wt and IL-37b-tg mice were stimulated with LPS for 4 hours; total RNAs from each group were isolated for microarray. A) Shows differentially expressed genes (DEGs) that are down regulated by IL-37b (the red area); the overlap area is common genes between IL-37b and wt; while the green area are genes that are not affected by IL-37b. B) The red area shows DEGs that are specifically upregulated by IL-37b; the overlap area are the common genes between IL-37b and wt; while the green are genes that not affected by IL-37b.

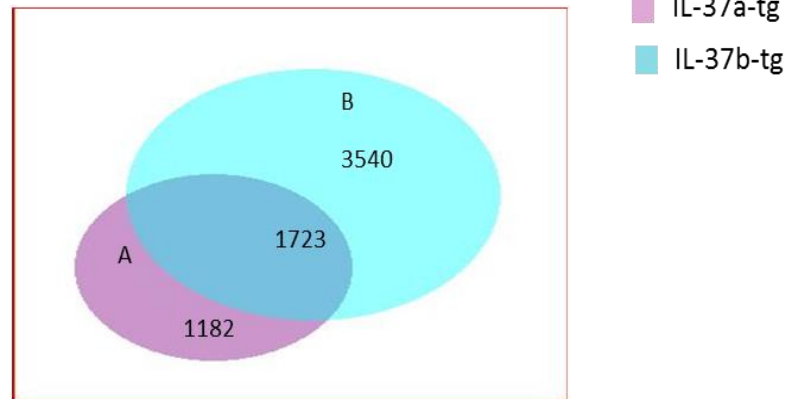
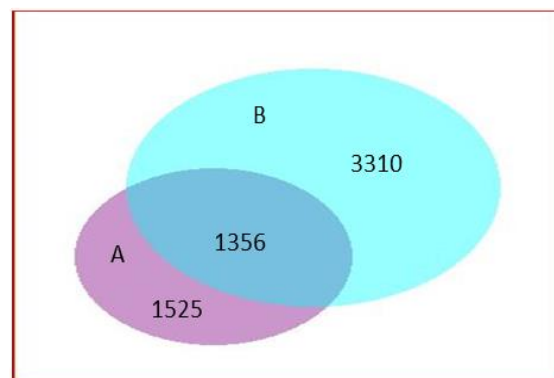
A: Wt > IL-37a-tg & IL-37b-tg**B: Wt < IL-37a-tg & IL-37b-tg**

Figure 5.3 Identification of common and specific genes differentially regulated by IL-37a and b in LPS stimulated spleenocytes

Spleenocyte cells from wt, IL-37a-tg and IL-37b-tg mice were stimulated with 500 ng LPS for 4 hours; cells harvested and RNA isolated for microarray assay. A) represents the genes that are down regulated by IL-37a and b; the pink area are genes down regulated by IL-37a only; the overlap area shows the genes that are down regulated by both IL-37a and b; while the blue area represents the genes that are down regulated by IL-37b only. B) shows the set of genes that are upregulated by IL-37a and b; specific genes upregulated by IL-37a shown in pink area; the overlap area are common genes, which are upregulated by both IL-37a and b; the blue area are genes that are upregulated specifically by IL-37b.

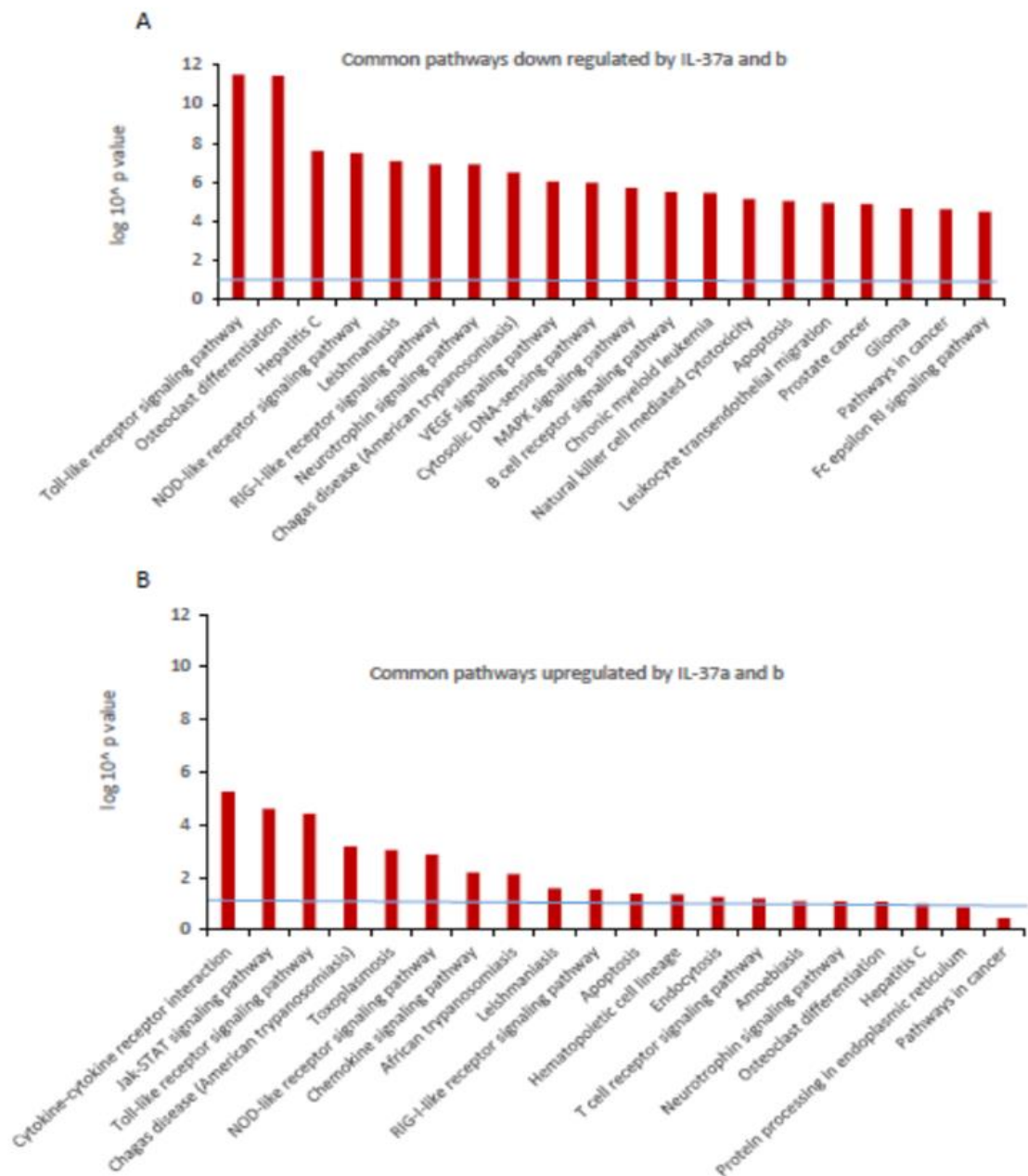


Figure 5.4 The signalling pathways regulated by both IL-37a and b in LPS stimulated spleenocytes.

The signalling pathways of the common genes regulated by both IL-37a and IL-37b in LPS-stimulated cells identified by the enrichment analysis on IDB and KEGG. The x-axis indicates the pathway name ; the y-axis is associated p-value presented by ($\log_{10}^{\text{pvalue}}$), the blue line implies the cut-off p-value (0.05). A) Represents common pathways down regulated by IL-37a and b. B) are the common pathways upregulated by IL-37a and b.

Table 5.1a Common pathways and related genes downregulated by both IL-37a and b

Pathway Name	Pathway Id	Pathway uploaded gene count	Genes in IDB for this entity	Pathway p-value	Pathway p-value (corrected)	Genes
Toll-like receptor signalling pathway	699	13	100	2.92E-12	2.30E-10	Cd14; Cxcl10; Ifnar1; Il1b; Map3k7; MAPK14; MAPK3; Nfkb1; Nfkb1a; Pik3cd; Rac1; TIRAP; Tollip;
NOD-like receptor signalling pathway	8111	8	57	3.13E-08	5.18E-07	Il18; Il1b; Map3k7; MAPK14; MAPK3; Nfkb1; Nfkb1a; Nod1;
MAPK signalling pathway	728	12	253	2.02E-06	1.67E-05	Cd14; Daxx; Il1b; MAP3k5; Map3k7; MAPK14; MAPK3; Nfkb1; Pla2g4a; Rac1; Relb; Tnfrsf1a;
B cell receptor signalling pathway	790	7	72	3.08E-06	2.42E-05	MAPK3; Nfkb1; Nfkb1a; Pik3cd; Plcg2; PTPN6; Rac1;
Chemokine signalling pathway	4351	9	195	5.30E-05	2.93E-04	Cxcl10; Cxcl16; Cxcr2; JAK3; MAPK3; Nfkb1; Nfkb1a; Pik3cd; Rac1;
Cytokine-cytokine receptor interaction	604	10	264	1.05E-04	5.27E-04	Cxcl10; Cxcl16; Cxcr2; Ifnar1; Il17a; Il18; Il1b; Kdr; Ltbr; Tnfrsf1a;
mTOR signalling pathway	743	4	61	0.002048	0.00602944	Igf1; Mapk3; Pik3cd; PTEN

The key pathways selected from the Pathways analysis presented in figure 5.4 A; these pathways are presented here in detail. Pathway Id is the number of the pathway in KEGG system; the name and number of the genes that are down regulated by IL-37a and b and involved pathway with P values.

Table 5.1b Common pathways and related genes upregulated by both IL-37a and b

Pathway Name	Pathway Id	Pathway uploaded gene	Genes in Innate DB for this entity	Pathway p-value	Pathway p-value (corrected)	Gene Symbols
Cytokine-cytokine receptor interaction	604	8	264	5.52E-06	1.60E-04	Csf1r; Cxcr3; Cxcr4; Cxcr6; Il12b; Il19; Il4ra; Il6st;
JAK-STAT signalling pathway	651	6	154	2.36E-05	3.05E-04	Il12b; Il19; Il4ra; Il6st; Stat6;
Toll-like receptor signalling pathway	699	5	100	3.71E-05	3.58E-04	Il12b; MYD88; Ticam1; TLR6; TLR7;
NOD-like receptor signalling pathway	8111	3	57	0.001345	0.00410596	Casp1; Naip2; Nlrp3;
Chemokine signalling pathway	4351	4	195	0.006492	0.01234527	Cxcr3; Cxcr4; Cxcr6; Stat3;

The key pathways selected from the pathways analysis presented in figure 5.4B; these pathways are presented here in detail. Pathway Id is the number of the pathway in KEGG system; the name and number of the genes that are upregulated by IL-37a and b and involved pathway with P values.

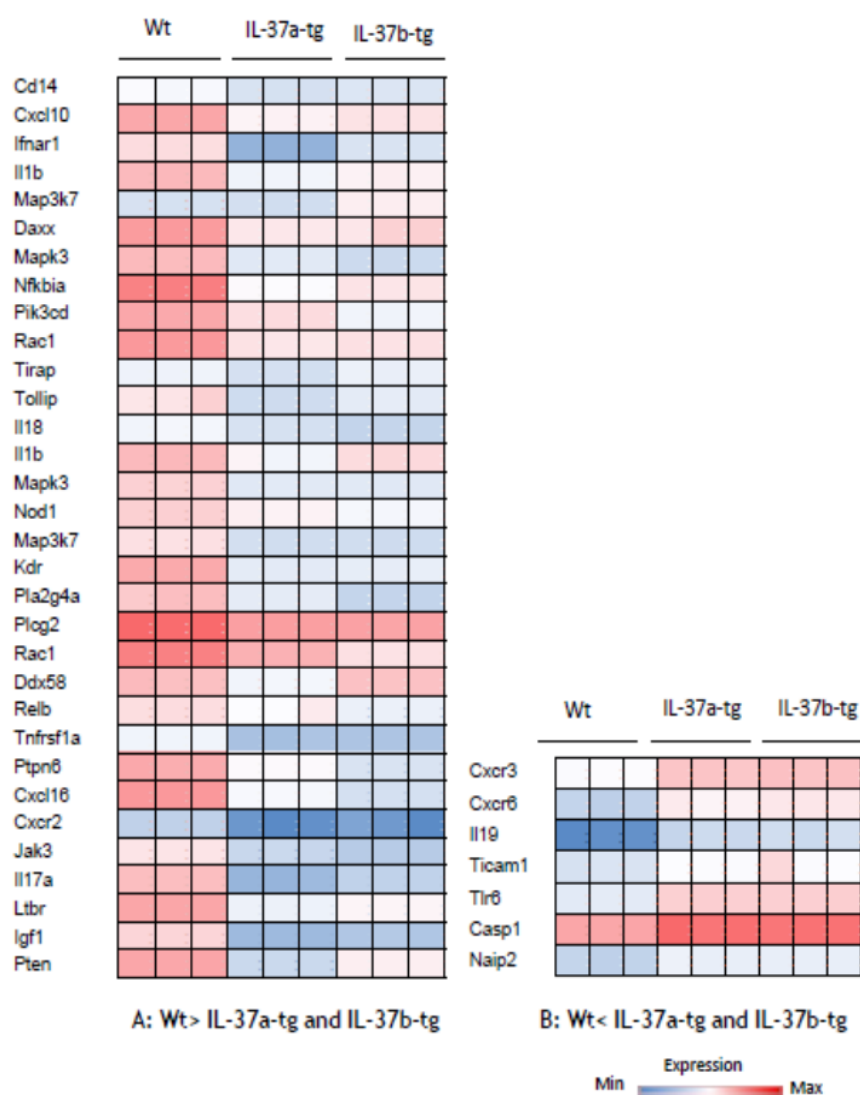


Figure 5.5 Heat maps presentation of the common genes regulated by IL-37a and b compared to wt control.

The representative common regulated genes by IL-37a and b selected from Tables 5.1 and 5.2 displayed in heat maps. The genes were analysed by microarray assay in triplicates. A) The representative genes (Table 5.1) that are down regulated by IL-37a and b; B) the representative genes (Table 5.2) that are upregulated by IL-37a and b compared with wt controls.

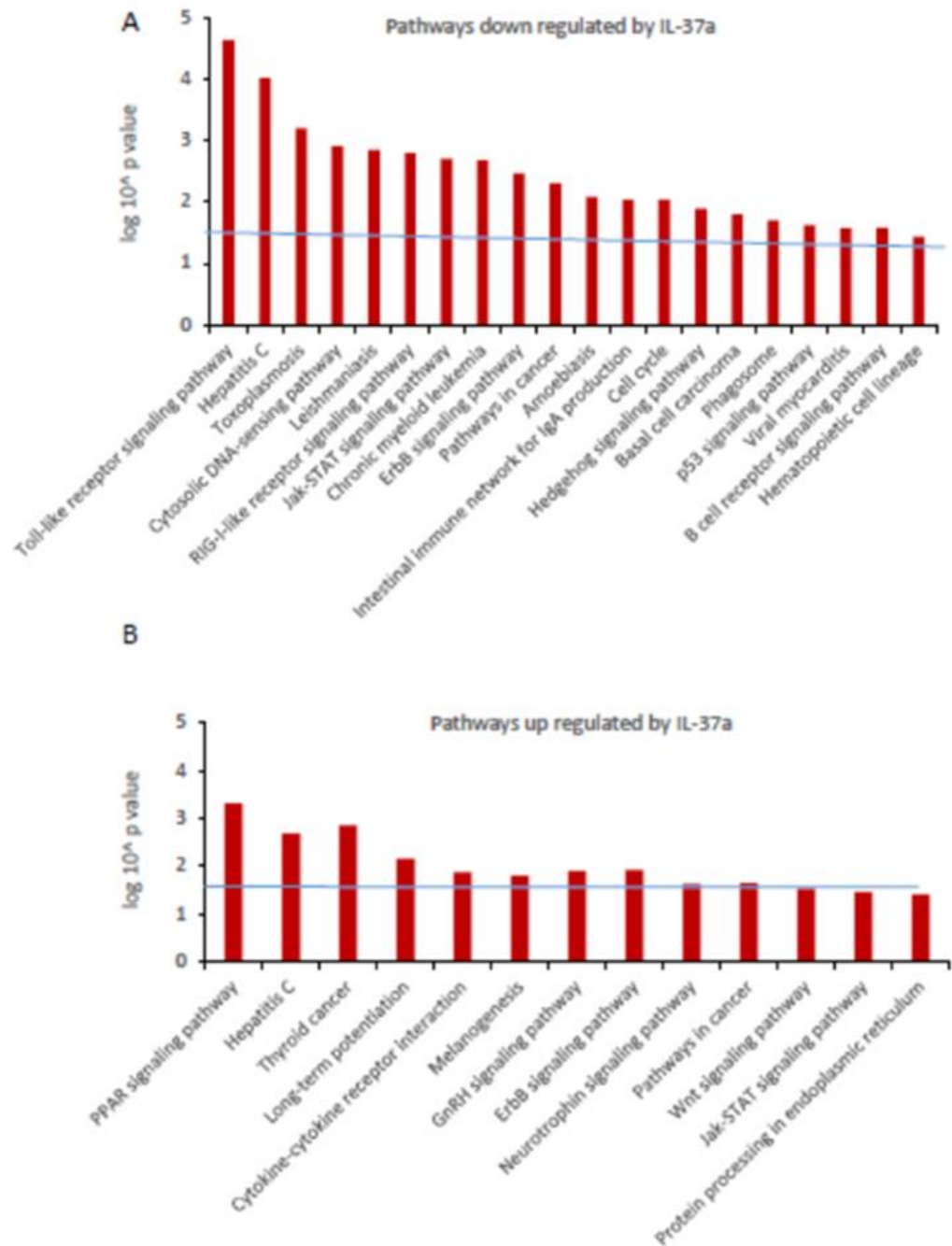


Figure 5.6 The signalling pathways selectively regulated by IL-37a in LPS stimulated splenocytes.

The signalling pathways of the genes selectively regulated by IL-37a in LPS-stimulated cells identified by the enrichment analysis on Innate DB and KEGG. A) Represents signalling pathways down regulated by IL-37a. B) The pathways upregulated by IL-37a.

Table 5.2 Signalling pathways and related genes specifically regulated by IL-37a.

(A)

Pathways down regulated by IL-37a	Pathway Id	Pathway uploaded gene count	Genes in InnateDB for this entity	Pathway p-value	Pathway p-value (corrected)	Gene Symbols
Toll-like receptor signalling pathway	699	5	100	2.33E-05	5.74E-04	Ifnb1; Ikbke; Irf7; Tlr1; Tlr2;
Jak-STAT signalling pathway	651	4	154	0.001979	0.008614	Cblc; Ifnb1; Il15; Jak2;
B cell receptor signalling pathway	790	2	72	0.026445	0.054359	Gsk3b; Pik3ap1;
Cytokine-cytokine receptor interaction	604	3	264	0.013364	0.043063	Ifnb1; Il15; Il1r2

(B)

Pathways up regulated By IL-37a	Pathway Id	Pathway uploaded gene count	Genes in InnateDB for this entity	Pathway p-value	Pathway p-value (corrected)	Gene Symbols
PPAR signalling pathway	696	3	82	0.000486	0.01408	Pitp; Pparg; Rxra
Cytokine-cytokine receptor interaction	604	3	264	0.013364	0.043063	Ccr3; Il13; Il20rb;
Wnt signalling pathway	698	2	141	0.029733	0.06159	Camk2a; Fzd1;
Jak-STAT signalling pathway	651	2	154	0.034973	0.067614	Il13; Il20rb;

The key pathways selected from the Pathways analysis presented in figure 5.6; Pathway Id is the number of the signalling pathway in KEGG system; the name and number of the genes in related pathway that are down regulated by IL-37a (A) and upregulated by IL-37a (B) with P values.

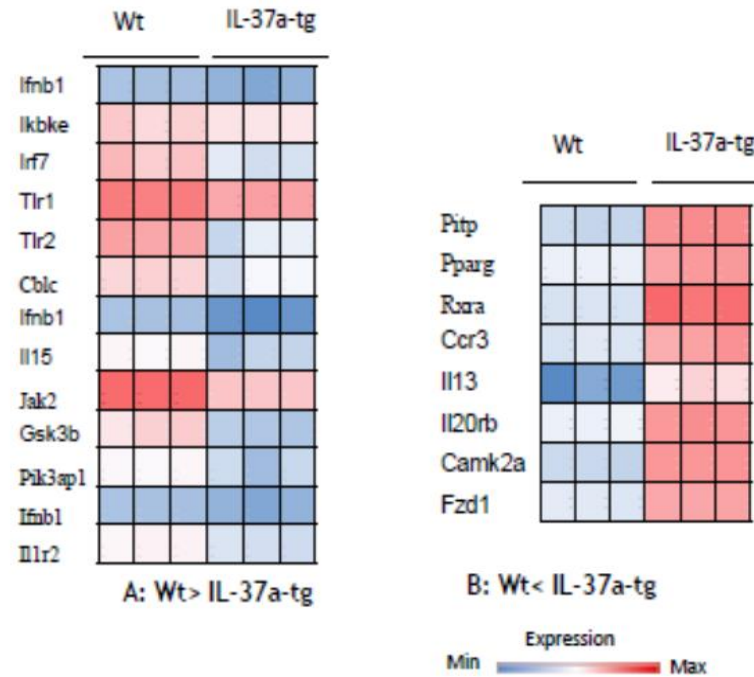


Figure 5.7 Heat maps presentation of the genes regulated specifically by IL-37a compared to wt control.

The representative genes specifically regulated by IL-37a selected from table 5.2 and displayed in the heat maps. The genes were analysed by microarray assay in triplicate. A) The representative genes from table 5.2A that are down regulated by IL-37a; B) the representative genes from table 5.2B that are upregulated by IL-37a compared with wt controls.

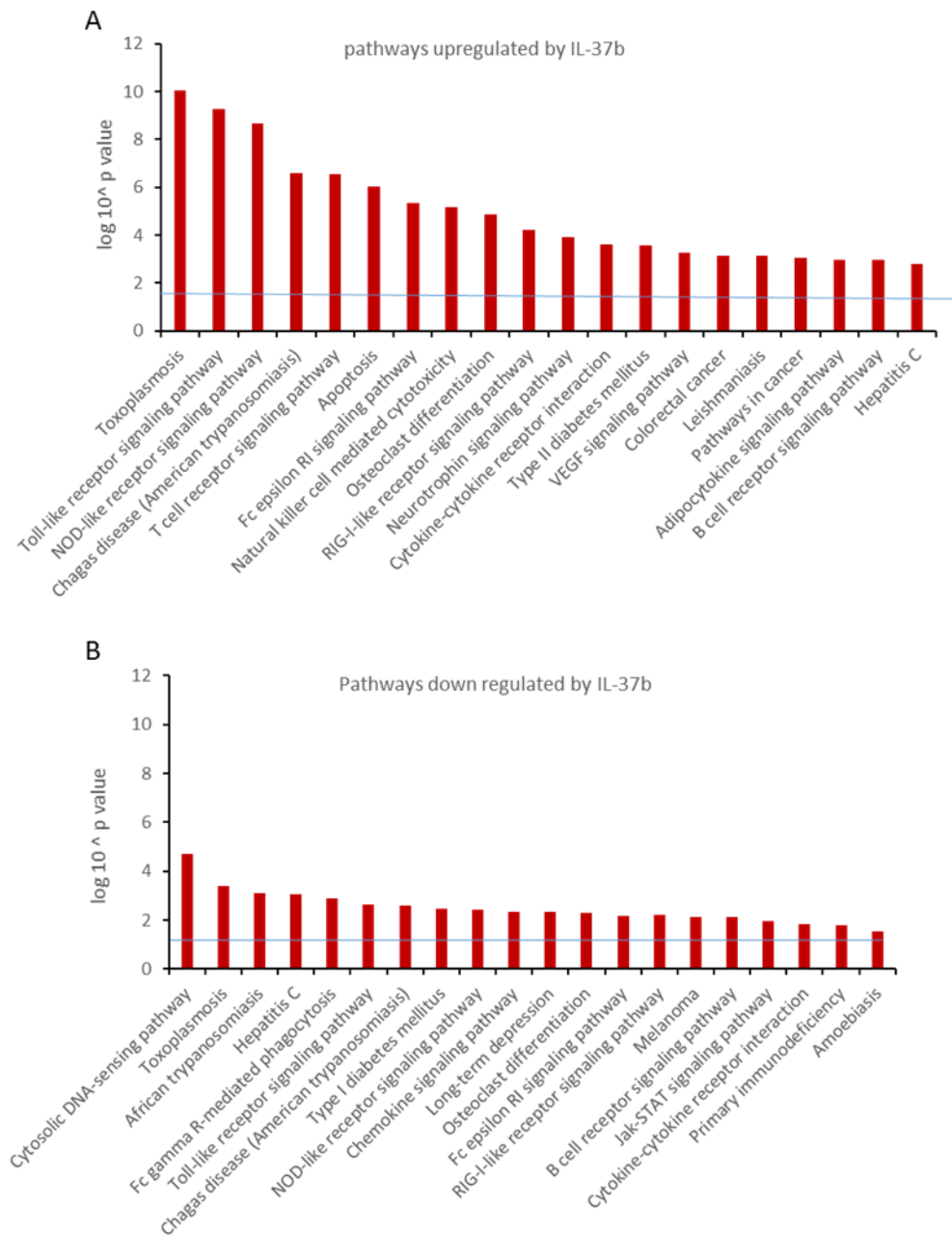


Figure 5.8 The signalling pathways selectively regulated by IL-37b in LPS stimulated splenocytes.

The signalling pathways of the genes selectively regulated by IL-37b in LPS-stimulated cells identified by the enrichment analysis on Innate DB and KEGG. A) Represents signalling pathways upregulated by IL-37b. B) The pathways down regulated by IL-37b.

Table 5.3 Signalling pathways and related genes specifically regulated by IL-37b

(A)

Pathway down regulated by IL-37b	Pathway Id	Pathway uploaded gene count	Genes in InnateDB for this entity	Pathway p-value	Pathway p-value (corrected)	Gene Symbols
Cytosolic DNA-sensing pathway	8116	5	61	2.06E-05	0.000714	Adar; Irf3; Mavs; Pycard; Zbp1;
Toll-like receptor signalling pathway	699	4	100	0.002265	0.016324	Akt1; Cxcl9; Il12a; Irf3;
NOD-like receptor signalling pathway	8111	3	57	0.003866	0.020901	Hsp90b1; Nod2; Pycard;
Chemokine signalling pathway	4351	5	195	0.004428	0.021278	Akt1; Cxcl9; Gnai2; Lyn; Was;
B cell receptor signalling pathway	790	3	72	0.007433	0.027955	Akt1; Inpp5d; Lyn;
Jak-STAT signalling pathway	651	4	154	0.010449	0.035445	Akt1; Ifng; Il12a; Socs1;
Cytokine-cytokine receptor interaction	604	5	264	0.015332	0.047365	Cxcl9; Ifng; Il12a; Il1r1; Tnfsf9;

(B)

Pathways up regulated by IL-37b	Pathway Id	Pathway uploaded genes	Genes in Innate DB for this entity	Pathway p-value	Pathway p-value (corrected)	Gene Symbols
Toll-like receptor signaling pathway	699	10	100	5.35E-10	3.28E-08	Casp8; Cd86; Irak4; Ly96; Map3k8; Mapk1; Mapk8; Pik3cg; Tlr4; Tnf;
NOD-like receptor signalling pathway	8111	8	57	2.11E-09	7.78E-08	Birc2; Card9; Casp8; Cxcl2; Mapk1; Mapk8; Naip5; Tnf;
T cell receptor signaling pathway	692	8	105	2.82E-07	6.48E-06	Cd8a; Csf2; Map3k8; Mapk1; Nfatc2; Nras; Pik3cg; Tnf;
Cytokine-cytokine receptor interaction	604	8	264	0.000244	0.001402	Csf2; Cxcl2; Il21; Il2rb; Tnf; Tnfrsf9; Tnfsf11; Vegfc;
B cell receptor signaling pathway	790	4	72	0.001106	0.004329	Mapk1; Nfatc2; Nras; Pik3cg;
Jak-STAT signaling pathway	651	5	154	0.002889	0.007935	Csf2; Il21; Il2rb; Pik3cg; Stat4;

The key pathways selected from the Pathways analysis presented in figure 5.8; pathway Id is the number of the signalling pathway in KEGG system; the name and number of the genes in related pathway that are regulated by IL-37b and a. A) Pathways down regulated by IL-37b; B) pathways upregulated by IL-37a.

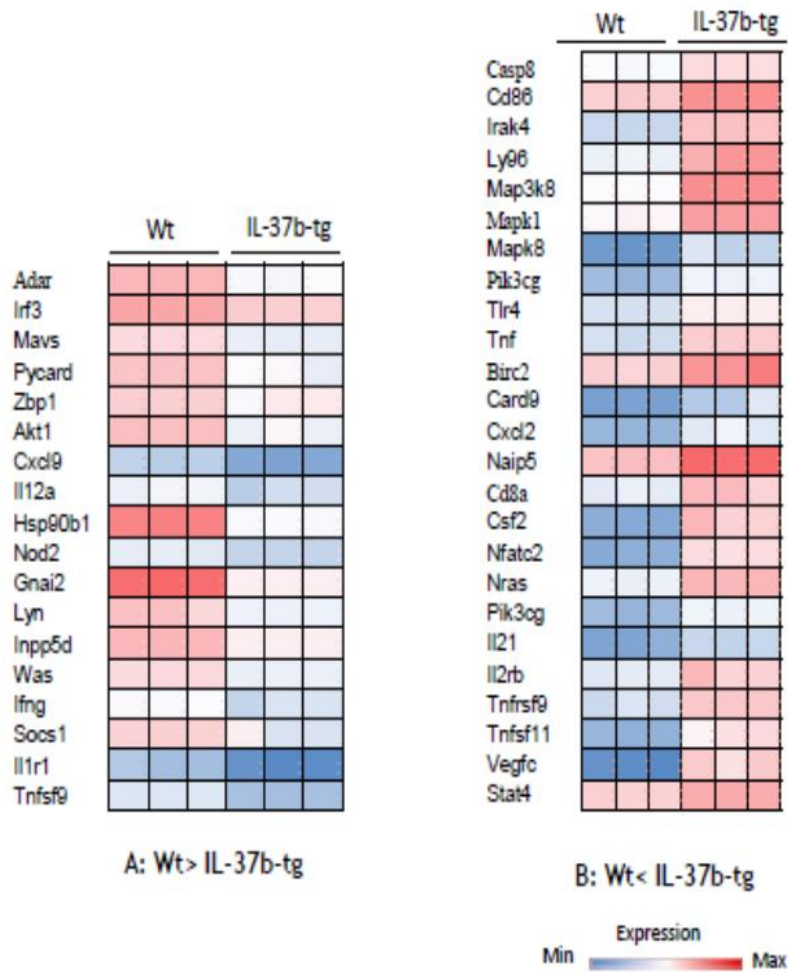


Figure 5.9 Heat maps presentation of the genes regulated specifically by IL-37b compared to wt control.

The representative genes specifically regulated by IL-37b selected from table 5.3 and displayed in the heat maps. The genes were analysed by microarray assay in triplicates. A) The representative genes from table 5.37A that are down regulated by IL-37b; B) the representative genes from table 5.3B that are upregulated by IL-37b compared with wt controls.

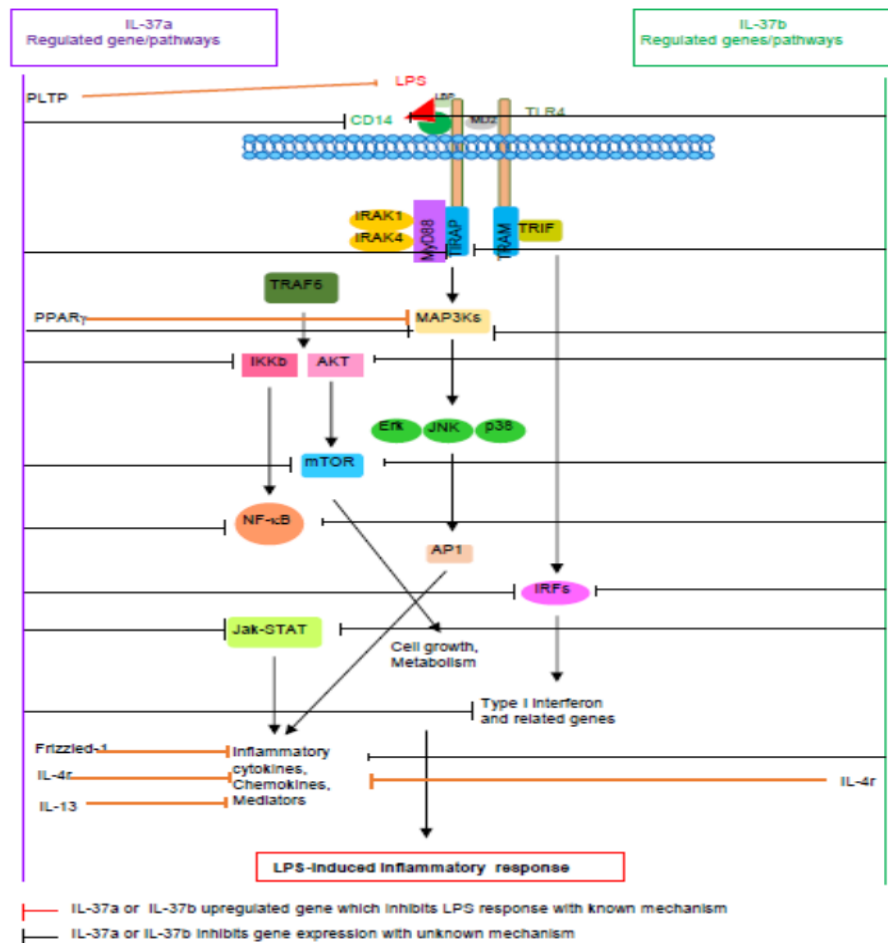


Figure 5.10 Proposed molecular mechanism by which IL-37a and IL-37b regulated TLR4 signalling.

IL-37a and b suppress TLR4 signalling through gene regulation of several pathways. Both IL-37a and b inhibit CD14 co-receptor, TIRAP, that affect LPS binding to TLR4 and signal initiation respectively, and inhibit MAPK, NF- κ B, PI3K, and mTOR pathways; and genes involved in Jak/STAT pathway. IL-37a specifically upregulate PLTP which affect LPS metabolism; PPAR γ ; Frizzled-1, IL-13, and IL4r that all promote anti-inflammatory function.

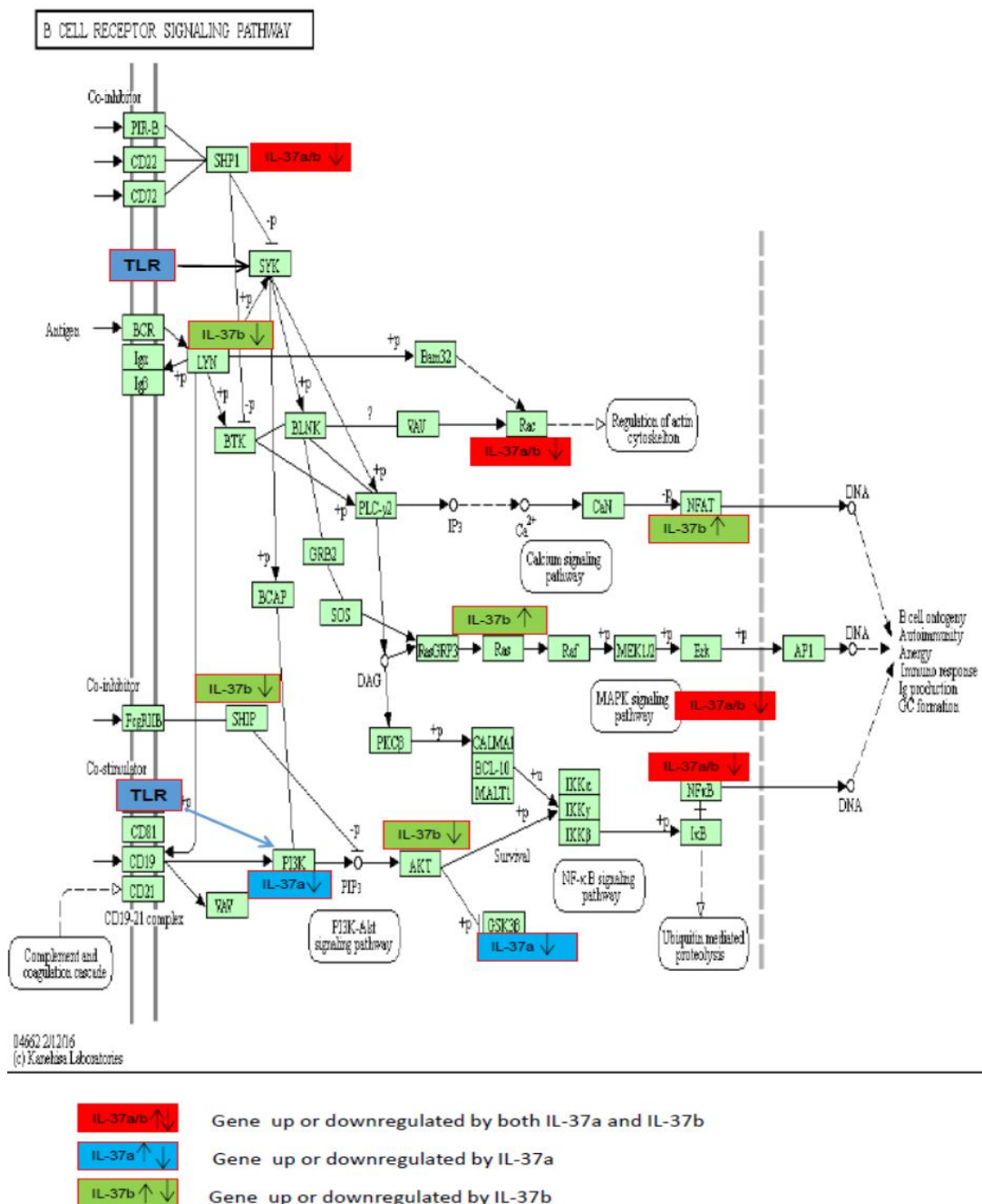


Figure 5.11 Proposed molecular mechanism by which IL-37a and IL-37b regulate TLR and BCR signalling in B cells

5.3 Discussion

In order to gain understanding of the similarity and difference between IL-37a and IL-37b in gene regulation, transcriptomic analysis was performed with LPS/TLR4-stimulated spleenocytes samples from wt control and IL-37a and IL-37b transgenic mice. The results demonstrated for the first time: 1) Compared to the wt control, IL-37a significantly down regulated 2905 and upregulated 2881 genes in the LPS-stimulated cells; indicating that IL-37a has a broad regulatory effect on global gene expression, not only suppressing but enhancing gene expressions. 2) Compared to IL-37b, IL-37a specifically inhibited 1182 and enhanced 1525 genes, suggesting that IL-37a differs from IL-37b in gene regulation. 3) By comparing with IL-37b, the common regulated genes between the two IL-37 isoforms were also identified, explaining the functional similarity between the two isoforms; 4) the common and unique signalling pathways between the two isoforms were also revealed using Innate Data Base (IDB) and KEGG enrichment analysis. These have provided molecular mechanism, at least in part, for the IL-37-mediated regulatory effect in our previous experimental observations.

Thus, the transcriptomic analysis further demonstrated that IL-37a is a previously unrecognised immunoregulatory cytokine and plays an important role in the regulation of TLR-signalling and beyond. In addition, IL-37a differs from IL-37b in gene regulation and signalling and may be a novel regulatory intervention for inflammatory diseases.

Consistent with our results that show IL-37a and IL-37b sharing functional similarity in the regulation of immune cell function (Chapter 4), a considerable amount of genes in LPS-stimulated cells were regulated by both IL-37a and IL-37b in the transcriptomics analysis. While the detailed mechanism still needs to be elucidated, because the two isoforms share the IL-1-like cytokine domain for receptor binding and signalling, it is mostly likely that IL-37a also signals via the same receptor as IL-37b and the common genes are regulated via the common IL-37 receptor-dependent pathway. Indeed, our recent results using IL-1R8 deficient cells demonstrated that the same as IL-37b, recombinant IL-37a also requires the receptor for signalling (Y. Ji, unpublished result). Apart from the common regulated genes, nearly half of the genes regulated by IL-37a and the

majority of genes regulated by IL-37b are specifically regulated by IL-37a or IL-37b, respectively. The result further demonstrates that IL-37a and IL-37b differ in gene regulation and signalling. Whereas it is poorly understood how the two IL-37 isoforms are differently involved in gene regulation, the unique genes may be largely attributed to their unique N-terminal sequence of the two IL-37 isoforms and their difference in nuclear translocation.

It has been shown that matured but not the FL-IL-37b is able to translocate into cell nucleus via Smad3 (Luo et al, 2017). Using the IL-37b transgenic mice lacking the IL-1R8, it was further demonstrated that nuclear located mature IL-37b can regulate LPS response in an IL-1R8-independent manner (Nold-Petry et al, 2015). While the detailed mechanism is still lacking, it has been reported that the genes regulated by the receptor-independent pathway of IL-37b are largely different from the ones regulated by the receptor-dependent pathway (Nold-Petry et al, 2015).

Because the FL-IL-37a contains a putative NLS and our preliminary results suggest that the majority FLIL-37a proteins translocate into cell nucleus via their NLS (R. Wei, unpublished result), we reason that the genes specifically regulated by IL-37a may be predominantly attributed to the nuclear FLIL-37a in a receptor-independent manner. Further experiments are needed to define the hypothesis by using the IL-37a transgenic mice without the IL-1R8. While the molecular targets and mechanism of action of nuclear IL-37a in the nucleus are still unknown and currently under investigation in our lab using Chip-sequence, it is known that the NLS containing N-terminal of IL-1a is sufficient to regulate cell function by translocation to the nucleus and binding to transcription activators and chromatin (Werman et al, 2004b). Since the putative elastase-matured IL-37a protein (R22-D192) shares 98% homology with the caspase-1 matured IL-37b (V46-D218), it is possible that after processing, matured IL-37a also translocate to the cell nucleus through the same Smad3-mediated mechanism as mature IL-37b. However, our preliminary result suggests that different from the FLIL-37, the majority of mature IL-37a proteins are located in the cell plasma, not cell nucleus (Wei, unpublished result). While the reason behind this is still unknown, it is likely that FL but not mature IL-37a is primarily responsible for the gene regulation in the cell nucleus. Nevertheless, our results suggest that FL-IL-37a is

a dual functional regulatory molecule, which regulates genes and cell function by different mechanism. Different from FL IL-37b; FL-IL-37a can serve as a nuclear regulatory factor when translocated into the cell nucleus. Similar to IL-37b, FL and mature IL-37a can also act as a cytokine when released from producing cells and trigger signalling via IL-37 receptor. Our transcriptomic analysis reveals that IL-37a and IL-37b control LPS/TLR4 signalling transduction by targeting multiple signalling pathways and via common and specific mechanisms (Fig 5-10): both isoforms inhibit the expression of TLR4 co-receptor CD14 and MyD88 adaptor protein TIRAP. IL-37a also induces the expression of LPS-binding protein PLTP to block LPS function; both these will prevent the initiation of LPS/TLR4 signalling. Both isoforms can further suppress the downstream TLR signalling, including MAPK, PI3K, mTOR, NF- κ B and JAK-STAT pathways. A part of the shared capacity of gene regulation, both isoforms can also act independently in gene modulation and control TLR signalling by different mechanisms. As such, IL-37a suppresses the expression of *Irf7* and *Ifnb1* in the TRIF-dependent pathway, on the other hand, IL-37b inhibited *Irf3*, altogether; this may lead to the down regulation of type I IFN production. Intriguingly, IL-37a, but not IL-37b, also increased expression of several immunoregulators with known anti-inflammatory activity, such as PPAR γ which inhibits TLR4-mediated inflammations (Wei R., unpublished) and Frizzled-1 which induces Wnt/ β -catenin signalling to promote anti-inflammatory function in macrophages (Wei R, unpublished). IL-37a also enhanced expression of the type II cytokine IL-13 and both IL-37 isoforms enhanced IL-4 receptor as well; these type II cytokines may counter regulate the expression and function of pro-inflammatory cytokines (Wynn, 2015). Altogether, these effects may impair the LPS-induced production of inflammatory cytokines, chemokines and mediators and also ameliorate inflamed leucocyte migration, cell metabolic and immune reactions, thereafter decreasing inflammatory response. Hence, the selective induction of IL-37a may represent a novel strategy for improving immunotolerance against dysregulated inflammatory response. In addition, this analysis also provides a possible molecular explanation for the IL-37-mediated regulatory effect on LPS-activated macrophages, monocytes and B-cells in (Chapter 4).

Our previous results showed that IL-37 signals increased IgG production and cellular proliferation in LPS-stimulated murine CD19⁺B cells (Chapter4). However, IL-37 inhibited inflammatory cytokine expression in murine and human B cells. The mechanism is still unknown (Chapter4).

Because the murine spleenocytes contain 40% B cells and the spleenocytes used for the transcriptomic study were stimulated with LPS, we therefore assessed the regulatory effect of IL-37 isoforms on TLR-stimulated B cells by analysing their gene profile in B cell signalling pathways. As illustrated in (Fig 5-11), our results suggested that IL-37a and IL-37b also positively or negatively regulate TLR and BCR signalling pathways in B cells, alone or together.

Although no clear picture can be drawn from this, probably due to the total spleenocytes used and the analysis being based on the IDB in which not all B cell-related genes are included, the result suggests the possible mechanisms underlying how IL-37 isoforms regulate TLR4/BCR signalling and function in B cells. Regarding the different effect of IL-37a and IL-37b on the regulation of MYD88 and TRIF-dependent pathway in B cell function, while; the precise signalling pathway is still lacking. TLR4 signals in B cells through at least two distinct pathways, one via the BCR leading to the activation of SYK, ERK, and AKT independent of MYD88, and the other through MYD88 leading to the activation of NF- κ B (Schweighoffer et al, 2017). The MYD88-independent pathway mainly mediated by TRIF-dependent pathway, and associated with the B cell proliferation and antibody production (Pihlgren et al, 2012; Yanagibashi et al, 2015).

However, the function of the MYD88-dependent pathway in this context was not investigated, but was likely involved in inflammatory cytokine production. Interestingly, we found that TRIF expression was enhanced, but the MYD88 adaptor TIRAP was inhibited by both IL-37a and b. The result suggests that IL-37a/b may enhance antibody but decrease inflammatory cytokine production by differentially regulated MYD88 and TRIF-dependent pathways. This hypothesis needs to be defined by using the B cells from MYD88 and TRIF deficient mice.

IL-37 may enhance B cell antibody production by suppressing the expression SHIP and SHP1 in B cell signalling.

BCR signalling is tightly regulated and mainly by SHP-1 and SHIP (Paessler et al, 2002). Mice deficiency in SHP-1 or SHIP all enhanced antibody production and autoimmune disease (Pao et al, 2007; O'Neill et al, 2011; Chen et al, 2017). We found that both IL-37a and b significantly suppressed the expression of PTPN6 encoding SHP-1, and IL-37b suppressed Inpp5d encoding SHIP in LPS-stimulated spleenocytes (Fig 5-11). Agreeing with the SHP1 down regulation, we found that IL-37b also enhanced effector molecules in the TLR4/SYK/ERK and NFAT pathways that are responsible for the TLR4-mediated IgG production in B cells, including MAPK1, Nras and Nfatc2, and the Pik3cg in PI3K pathway (Fig 5-11). Thus, down regulation of SHP-1 and/or SHIP may contribute to IL-37-enhanced antibody production and proliferation in B cells. However, the impact and underlying mechanism by which IL-37 down regulates SHP-1 and SHIP in B cells is currently unknown. Further study will be important using B cells with the two phosphatases knock downed to evaluate the importance of the down regulation of the phosphatases in the IL-37-enhanced antibody production and B cell proliferation.

The expression of SHIP but not SHP-1 has been examined above in Chapter 4. The result showed that the SHIP expression in LPS-stimulated B cells from IL-37a-tg mice was significantly lower than that in wt cells. While this suggests that IL-37a can also regulate SHIP, the result is contradicted by the transcriptomic result. The reason is unclear, maybe due to the difference in cell population, the earlier study using purified B cells the later one the spleenocytes, and to also the culture conditions (4 hours vs 3 days).

The possible mechanism by which IL-37 suppresses LPS-induced inflammatory cytokine production in B cells.

While the down regulation of inhibitory SHP-1; and SHIP may provide, at least in part, explanation for the IL-37-enhanced antibody production in murine B cells, it fails to explain why IL-37 is still capable of inhibiting inflammatory cytokine production in B cells. Studies have also showed SHIP-1 deficiency elevating NF- κ B-dependent inflammatory cytokine and MAP kinase activation exploiting TLR4-independent PI3K/Akt pathway (Li et al, 2016). By analysing the genes regulated by IL-37a and b in BCR signalling pathway, we found that both IL-37 are able to down regulate expression of key genes in NF- κ B pathway in B cells, alone or

together: IL-37a down regulated *pik3ap1* in PI3K pathway and IL-37b down regulated the important *Lyn* and *Akt*. Both isoforms also down regulated *pik3cd* in PI3K pathway, *Nfkb1* and *Nfkb1a* in NF- κ B pathway as well as MAPK3 in MAPK pathway (Fig 5-11). Thus, it is possible that the subsequent increasing NF- κ B activation resulting from the SHP-1 and SHIP down regulation by IL-37 will be reduced quickly by the direct inhibitory effect of IL-37 on the elements in the signalling pathway as did in TLR signalling. However, this needs to be confirmed in phosphorylated protein levels by western blot in B cells stimulated or not stimulated with IL-37. Based on these preliminary results, my hypothesis is that the down regulation of B-cell regulators SHP1 and SHIP will be mainly responsible for the IL-37a/b-mediated antibody response and that the suppression of NF- κ B and MAPK pathway by IL-37a/b may result in the impaired inflammatory cytokine production in B cells. Obviously, it is worthwhile to test the hypothesis in vitro and in animal model in vivo. The transcriptomic experiment has some limitations and could be improved; it will be ideal to have more LPS stimulation time points in B cells in order to see kinetic changes in gene expression, in particular the early time 2-3 hours after stimulation to see the transcriptomic changes in the initiation stage of B cell activation. The important differentially expressed genes need to be validated by qPCR with specific primers and in protein levels by western blot, FACS or ELISA. Their function in IL-37-mediated effect also needs to be evaluated by siRNA knockdown or overexpression in immune cells. The transcriptomics study also shows that IL-37a and b may have global regulatory effect on other diseases beyond LPS-induced inflammation. This will be discussed in the General Discussion.

Chapter 6

General Discussion

6 General discussion

6.1 IL-37 family: why do we need 5 isoforms?

IL-37 is a new member of the IL-1 family and consists of 5 isoforms (IL-37a, b, c, d, and e). Until now, only the IL-37b isoform has been studied in detail. While IL-37b is important in immune regulation and tolerance, the biology and function of other isoforms are largely unknown. From an evolution point of view, if a gene is no longer needed, it will be deleted or become a pseudogene. Thus, it is important to know the function of other IL-37 isoforms. Based on the information in the general introduction of IL-37 isoforms (Chapter 1) and the results from our own lab, here I will mainly discuss their differences in the structure, induction, regulation, tissue distribution and potential function. By doing so, hopefully we can answer the question, at least in part, why we need five IL-37 isoforms.

6.1.1 IL-37 isoforms are different in gene organisation and protein levels:

IL-37 gene contains 6 exons and encodes five isoforms due to alternative gene splicing (Boraschi et al, 2011). Pro-IL-37a is a protein with 192 amino acids encoded by exon 3 to 6. It contains a unique nuclear localization sequence (NLS) in the N- terminal for importing from cytoplasm into the cell nucleus. The pro protein also contains a unique elastase cleavage site and can be digested by elastase to form mature protein, while the other IL-37 members carry a caspase-1 cleavage site (Boraschi et al, 2011). Pro IL-37b is the biggest protein in the family consisting of 218 amino acids long encoded by all the exons, except the exon 3 and can be cleaved by caspase-1 to produce mature IL-37b (Pan et al, 2000). IL-37d is encoded by exon 1 and exons 4 to 6 to give a 197 amino acid pro protein and can also be processed by caspase-1. IL-37c is 178 amino acid long encoded by exons 1, 2, 5 and 6 and IL-37e is 157 amino acids long encoded by exons 1, 5 and 6 (Boraschi et al, 2011). Some IL-37 isoforms also contain a key IL-1-like β -trefoil structure for receptor binding and signalling, which is encoded by the exons 4-6. Based on whether or not carrying the IL-1-like domain, the isoforms can be divided into two functional groups; the isoforms a, b, and d (i.e. those encoded by the complete exons 4-6) will therefore be expected to bind to the same receptor and trigger the same range of biological effects. In contrast,

IL-37c and e lack the key exon 4 and are expected to give rise to a partial IL-1-like β -trefoil structure and their functions are unknown (Boraschi et al, 2011). This protein/structural evidence suggests that the isoforms may vary in induction and function.

6.1.2 Difference of IL-37 isoforms in induction and expression

One possibility for why we need five IL-37 isoforms is that these isoforms may be selectively expressed in different organs or differently induced in different cell/tissues in disease by different stimuli. Indeed, the tissue specific expression of the IL-37 isoforms has been reported (Taylor et al, 2002). While they are expressed in some common organs, IL-37a is the only isoform that is expressed in the brain, IL-37b is the only one present in kidney, IL-37c is the heart-specific isoform, IL-37d and e are only expressed in bone marrow and testis, suggesting that IL-37 isoforms may have different effect in organs.

Current evidence also suggests that IL-37 gene expression is highly regulated. IL-37 genes contain an instability element (A-rich homology box) in exon 5, this makes the IL-37 mRNA unstable (Bufleret al, 2004). The function of instability elements can be overcome by inflammatory stimuli to ensure the cytokine genes can only be increased in inflammatory, but not in the homeostasis conditions. As such, resting cells only produce low levels of IL-37b proteins and the expression can be upregulated by an array of inflammatory stimuli and cytokines (TLR agonists, IL-1 α , IL-18, TNF- α , IFN γ) (Nold-Petry et al, 2015). Thus, it is important to know how other IL-37 isoforms are regulated and whether they are regulated in the same or different ways in immunity and diseases. However, the knowledge in this area is still very poor, mainly because the potential importance of IL-37 isoforms has not been fully appreciated.

There is only one report which determined the expressions of IL-37 isoform in human PBMCs (Rudloff et al, 2016). The results show that IL-37 isoform expressions are differently regulated by different TLR agonists within 8 hours cultures. TLR4 ligand LPS upregulates IL-37b and c in this experimental condition. IL-37c expression is predominantly induced by pam3 (TLR2 ligand); while TLR7/8 agonists had no effect on the induction of IL-37 isoform expression. The authors also showed that the expression of IL-37a and e was not

upregulated with any of these stimuli in this experiment. While it was the first report on the regulation of IL-37 isoform expression in cell level and sheds new insight for the difference of IL-37 isoforms in induction, this study has some drawbacks; the study on time effect was not long enough, the induction of IL-37 isoform expression beyond 8 hours has not been studied. In addition, the details of the qPCR primer sequence for each of the IL-37 isoforms used in the study were not provided; this makes it difficult to interpret the real value of the result. We further studied time and dose effect of TLR agonist on the IL37 isoform expression and found that the induction of IL-37 isoforms is differing depending on dose and time (Chapter 4). We also found that IL-37a is one of the highly induced isoforms.

6.1.3 Functional differences of IL-37 isoforms

While IL-37 (*IL1F7*) gene has been identified 10 years ago, its key biological function has just emerged recently. So far, among the isoforms, only the function of IL-37b and IL-37d has been reported.

Current evident suggests that IL-37b is a new member in the portfolio of classical anti-inflammatory cytokines, including IL-10 and TGF- β (Conti et al, 2016). However, different from IL-10 and TGF- β , these global immune suppressors, IL-37bis inflammatory signal induced and feed back inhibits the ongoing inflammatory response in vitro and in vivo. IL-37b is a dual functional cytokine and suppresses inflammatory response via the receptor-dependent and independent mechanisms; with the receptor-dependent manner, the secreted IL-37b binds to receptor complex IL-18Ra and IL-1R8 and elicit anti-inflammatory signalling (Nold-Petry et al, 2015; Dinarello et al, 2016). With the receptor-independent manner, the mature but not the FL-IL-37b can interact with Smad3, translocate into the cell nucleus and thereby facilitates the cytokine-suppressing properties through a Smad3-dependent mechanism. The importance and detailed mechanism underlying the receptor-independent regulatory effect of IL-37b is largely unknown, whereas the IL-37b-mediated protective effect against LPS-induced endotoxic shock is receptor-dependent (Jia et al, 2018).

It has been reported recently that IL-37dis also bioactive (Zhao et al, 2018). The same as IL-37b, IL-37d can also suppress inflammatory response via Smad3-

dependent mechanism. However, different from IL-37b, IL-37d fails to induce signal via the IL-37 receptor. Since IL-37d and b share the common C- terminal sequence and the IL-1 like domain it is difficult to understand why IL-37d is unable to signal via the receptor. The function of IL-37a has not been reported and this is the current focus in our lab. We found so far that pro-IL-37a is also dual functional and can serve as a nuclear regulator in the cell and a cytokine when secreted out of the producing cells. Different from other isoforms, pro IL-37a carries a unique putative NLS domain that may allow it to translocate into cell nucleus. Our result confirms its nuclear location; and further demonstrated that nuclear IL-37a can regulate gene expression (Xu, unpublished result). We also found that IL-37a possesses a similar C-terminal structure as IL-37b and can also bind to the same receptor as IL-37b and trigger the similar range of biological effects. However, we expect the unique dual effect of IL-37a may render IL-37a a super regulatory effect in immunity and disease compared to IL-37b and d. This is currently under investigation in the lab.

Nothing is known about the function of the two IL-37 isoforms lacking the full IL-1-like domain. Since the 12 B-trefoils in the IL-1-like domain are required for the receptor bind and signal in cytokines in IL-1 family, and both IL-37c and e lack the first three B-strands in the B-trefoil structure, it has been speculated that the IL-37c and e may not be able to bind to the receptor; and therefore lack the cytokine activity (Nold et al, 2010; Ellisdon et al, 2017). However, they may regulate other IL-37 isoforms function by competing or interfering with their mRNA splicing. As all pro IL-37 isoforms, except pro IL-37a, contain the caspase-1 cleavage site and can be processed into mature protein by the same enzyme, IL-37c and e may also affect other isoforms maturation and function by competing with the enzyme in the microenvironment in vivo. To support the hypothesis, all cytokines in IL-1 family are self-regulated, for example, IL-1 is regulated by IL-1 antagonist, IL-18 is via a binding protein (IL-18 BP), and IL-33 by the soluble IL-33 receptor (Hayakawa et al, 2007; Dinarello et al, 2013). Alternatively, while unable to induce receptor-dependent effect, IL-37c and e may also be able to regulate gene expression as IL-37b and d via Smad3 to translocate into cell nucleus.

6.1.4 Conclusion and future directions

In summary, the five IL-37 isoforms are different in protein structure, induction and function as well. Three of them have been demonstrated to play an important immunoregulatory role in disease and tolerance. These IL-37 isoforms may be therapeutically important in the treatment of a broad range of chronic disorders, including inflammatory and autoimmune diseases. However, much remains to be studied: it is still unclear whether the isoforms regulate inflammation with same or different effect. The precise mechanisms by which IL-37 isoforms suppress inflammatory response are still largely unknown. The different involvement and interaction of IL-37 isoforms in a given disease context need to be determined. Furthermore, while they are likely bioactive, the function of IL-37c and e isoforms remains unclear. Finally, the importance and relationship of IL-37 with other immunoregulatory cytokine, including IL-10 and TGF- β in immune tolerance need to be evaluated in the future. Nevertheless, it is possible that the IL-37 isoforms will synergise, back up or balance each other's function in the regulation of inflammation, thus ensuring that an ongoing inflammatory response is appropriately controlled.

6.2 IL-37 an immune inhibitor or regulator?

IL-37b has been originally characterised as a fundamental inhibitor of innate immune response (Nold-Petry et al, 2010). Later research confirms the hypothesis on innate immune cells and further reveals that IL-37 not only inhibits innate immune cells but also T-cells as well, indicating that IL-37 may inhibit both innate and adaptive immunity. Furthermore, all the current evidence suggests that IL-37 is an immune inhibitor and inhibits immune cell functions via both direct and indirect mechanisms. However, our results strongly suggest that IL-37 can also enhance immune cell function, at least in B cells. This has raised an important question: whether IL-37 is an immune inhibitor or a regulator. In this part of the general discussion, I sought to answer the question by collecting and analysing all the evidence supporting both proposals.

6.2.1 Evidence supporting IL-37 as an immune inhibitor

Much research supports the proposal, which demonstrated that IL-37 can inhibit both innate and adaptive immune cell functions. IL-37 is expressed in a wide

range of immune cells, including monocyte, macrophages, mast and T cells, and IL-37 plays an inhibitory role in the immune cell functions. It has been demonstrated that monocytes express highest levels of IL-37 among the human immune cells (Rudloff et al, 2016). This suggests that monocytes may be a primary source of IL-37 in immune response. It is also reported that IL-37 is inducible in monocytes by mycobacteria in tuberculosis (TB) patients; the expression is negatively correlated with the levels of inflammatory cytokines IL-1 β , IL-6 and TNF- α (Zhang et al, 2016). However, the detailed function of IL-37 on monocytes has not been explored. Its effect on macrophages has been well studied, including our own study. IL-37 can effectively suppress TLR signalling induced inflammatory cytokine production (Nold-Petry et al, 2015; Huang et al, 2016). In oxidized low-density lipoprotein (ox-LDL)-activation conditions, IL-37b effectively inhibited the production of IFN γ , TNF- α and NO in the macrophage (Huang et al, 2016).

The mast cells play a pathogenic role in many conditions, including asthma, allergy and psoriasis (Conti, 2017). Several studies show that IL-37b is able to suppress mast cell migration and function in murine model of psoriasis (Teng et al, 2014) and asthma (Conti et al, 2016).

The influence of IL-37b on adaptive immune cells has also been assessed and only T cells have been studied so far. It has been shown that IL37 is expressed by CD3 $^{+}$ and CD4 $^{+}$ T cells and can inhibit Th17 cell function (Ye et al, 2014). In Th17 polarisation condition, IL-37 effectively inhibits IL-17 expression and Th17 cell proliferation but not the differentiation from naive human CD4 $^{+}$ T cells.

The study of regulatory effect of IL-37a and IL-37d has just begun. The only report shows that IL-37d is expressed in peripheral blood mononuclear cells (PBMCs), epithelia and umbilical cord-derived mesenchymal stem cells (UCMSCs). IL-37d overexpression inhibits inflammatory cytokine IL-1 β induced IL-6 production in epithelia cell line and macrophages (Zhao et al, 2018). However, its role in the regulation of other immune cell function has not been studied yet. Our results demonstrated that IL-37a is also bioactive and can suppress LPS-stimulated inflammatory cytokine production in human and murine macrophage and monocytes (Xu, unpublished results). IL-37a can also regulate B cell function and this will be discussed below. Its effect on other innate and adaptive immune

cell function is currently under investigation. While the precise mechanisms are still poorly understood, IL-37 can suppress innate and adaptive immune cell function directly or indirectly.

6.2.1.1 The direct inhibitory effect of IL-37 on immune cell activity

The direct effect of IL-37 can perform in autocrine or paracrine manner; in the autocrine, an immune cell produces the cytokine first, the cytokine then regulates the function of the producer cell via receptor dependent or independent pathways. As discussed before, IL-37a, b and d can also enter the cell nucleus and regulate cell function as a nuclear factor without the receptor. However, the detailed mechanism by which nuclear IL-37 isoforms regulate gene function is largely unknown. In contrast, the receptor-dependent regulatory effects of IL-37 isoforms are relatively well studied; the secreted free IL-37 and IL-37a, but not IL-37d can bind to its receptor and regulate cell function via receptor-mediated signalling (Nold-Petry et al, 2015; Zhao et al, 2018 M). This was evidenced by the lost inhibitory effect of endogenous or recombinant IL-37 in IL-37 receptor knockout mice or the mice treated with anti-IL-37 receptor antibody (Nold-Petry et al, 2015).

In the paracrine, the IL-37 secreted by one immune cell type, for instance, monocytes can suppress the function of other surrounding immune cells that possess the IL-37 receptor. This will allow an enlargement of IL-37-mediated effect in immune response in tissue and organ. However, the indirect inhibitory effect mediated by IL-37 on immune cells with the indirect arm, IL-37 may control immune activation by downregulating antigen-presenting cell (APC) function and/or by inducing regulatory T cells (Treg) and tolerogenic dendritic cells (tDC). This could lead to a systemic impact of IL-37 on the ongoing inflammatory response.

Effect on APC function: DC is a key APC in tissues and plays an important role in the initiation of immune response. It has been shown that IL-37 can inhibit DC maturation and antigen presentation. For example, the LPS-stimulated maturation and activation of DC isolated from IL-37b-tg mice was markedly reduced (Luo et al, 2014). This was evidenced by the IL-37 treated DC reduced expression of MHC II and costimulatory molecule CD40 and the reduced

production of inflammatory cytokines, IL-1 β , IL-6, and IL-12 (Luo et al, 2014). More importantly, the IL-37b-treated DC failed to effectively activate naive T cells (Luo Y, et al, 2014). Thus, control of DC function by IL-37b will affect the development of antigen-specific immune activation. Treg, in particular the Forkhead box proteins-3 (FOXP3⁺)Treg, are fundamentally required for the control of development of inflammatory and autoimmune disease (Long and Buckner, 2011). In order to elicit a maximum and systemic regulatory effect, IL-37 can also promote the Treg development but suppress antigen-presenting cell (APC) function. It has been reported that T cells also express IL-37 and IL-37 differently modulates the function of CD4⁺ T cell subsets. IL-37 treatment selectively decreases Th1 and Th17 cell development but increases Treg cell polarisation and function (Ji et al, 2017). CD4⁺ Treg also express IL-37 and IL-37 promotes the expression of Treg transcript factor fork head/winged helix transcription factor p3 (FOXP3) and the suppressive cytokines such as TGF- β and IL-10 in Treg (Shuai et al, 2015). Furthermore, silencing the IL-37 gene in human Treg impairs the suppressive function of Treg (Shuai et al, 2015).

IL-37 may also control inflammatory response via the induction of tolerogenic tDC. The tDC is a subset of DC and plays a regulatory role in immune tolerance. Different from other DC, tDC is characterised by its ability of immune suppression and the polarisation of Treg (Chang et al, 2017). It has been demonstrated that the DC isolated from IL-37 transgenic mice expresses high level of IL-10 and reduced its ability in the activation of antigen-specific T cells, suggesting that the DC is the tDC. Furthermore, the tDC can polarise Treg and suppress the CD8 cytotoxic function in vitro and in contact hypersensitivity in mice (Luo et al, 2014). Thus, by inhibiting APC and promoting Treg and tDC development and functions, IL-37 could maximise its systemic regulatory effect in order to more effectively control inflammatory response. However, the potential effect of other IL-37 isoforms in the regulation of APC, Treg and tDC is still unknown.

6.2.1.2 IL-37 can enhance function of some immune cell sets

Whereas the inhibitory effect of IL-37 in immunity and in disease has been well documented, the immune enhancement effect of IL-37 has been largely overlooked. By review and analysis of the results from our and other labs, there

is good evidence demonstrated that IL-37 can also effectively promote immune cell functions.

We found that IL-37a can promote B cell antibody production and proliferation (Chapter 4). The effect is not limited to IL-37a, as IL-37b is also be able to do so. Thus, it is likely that enhanced B cell function is a general phenomenon for IL-37.

As discussed above, while IL-37 decreases the function and development of effector T cell, such as Th1 and Th17 cells, it selectively increases Treg cell polarisation and function (Ji et al, 2017). Treg express IL-37 and IL-37 enhances the expression of Treg transcript factor FOXP3 and the suppressive cytokines TGF- β and IL-10 in Treg (Shuai et al, 2015). It will be important to see if other IL-37 isoforms have same effect on Treg.

Macrophages can be further polarised into M1 or M2 subtypes (Martinez and Gordon, 2014). The M1 macrophages play an important role in infection and inflammation. They are characterised by the expression of iNOS and the production of NO and pro-inflammatory cytokines, including IL-1 β and IL-12 (Castleberry et al, 2008). The M2 macrophages, on the other hand, are involved in immunoregulation and tissue repair by producing IL-10, TGF- β , arginase 1 (ARG1) and inhibit NO production. It has been reported that IL-37 increases the development of M2 macrophage in mycobacterium tuberculosis infected macrophages by enhancing the expression of ARG1, TGF- β , IL-10 and reducing NO production in the macrophages (Huang et al, 2015). Consistently, IL-37b also effectively inhibits the ox-LDL-induced M1 macrophage development; and turned the cells into M2 cells (Huang et al, 2016). This demonstrated that IL-37 can selectively polarise M2 macrophages and may be involved in tissue repair and homeostasis.

6.2.2 Transcriptomic evidence supports that IL-37 can enhance immune response

The transcriptomic (RNA sequencing) results from other and our own lab also demonstrated the enhancement effect of IL-37 in transcriptome in immune cells. It has been reported that IL-37b predominantly upregulated the expression of

many genes, while it is also able to down regulate gene expression in the LPS-stimulated spleenocytes from IL-37b transgenic (Nold-Petry et al, 2015). Genes were highly upregulated and only 448 genes were downregulated in LPS stimulated IL-37-tg spleenocytes compared to the wt controls, suggesting that IL-37b mainly enhanced gene expression in the LPS-stimulated cells. Unfortunately, the nature and related signal pathways of the enhanced genes have not been provided in the report. Instead, the authors focused on only the 139 genes associated with innate immune response and showed that among these genes, 46% were increased by IL-37b in the context. By further analysing the genes upregulated by IL-37b, they found that while some of the genes encoded inhibitors of inflammation, others are closely associated with inflammatory response, for example, the *Tnfa* gene, TRAIIP (a TLR adaptor), *Plcd3* (Nold-Petry et al, 2015), S100a (a TLR4 agonist) and Pycard (Nold-Petry et al, 2015). However, the significance of the upregulated inflammatory genes in IL-37-mediated effect in the immune context has not been explored. Nonetheless, these results demonstrated that IL-37b not only inhibits but also enhances gene expression in immune cells.

We also studied the transcriptome of both IL-37a and IL-37b in the LPS-activated spleenocytes from wt, IL-37a or IL-37b transgenic mice (Chapter 5). Our own transcriptomic results show that, firstly, consistent with the published result, 5263 genes were upregulated and 4666 genes down regulated by IL-37b when compared to the wt control. More importantly, compared to the wt control, IL-37a also significantly upregulated 2881 and down regulated 2905 genes in the LPS-stimulated cells, indicating that IL-37a also has a broad regulatory effect on global gene expression, not only suppressing but also enhancing gene expressions.

The gene ontology study of these common upregulated genes by both IL-37a and IL-37b reveals that both the cytokines significantly upregulated genes in at least 10 pathways (Fig 5-4B, Chapter 5). These enhanced key pathways include cytokine-cytokine receptor interaction, JAK-STAT, TLR, NLR, chemokine, apoptosis, endocytosis, and the TCR. While the impact of the upregulated genes and pathways is largely unknown, given the importance of these pathways in

immune activation, these IL-37-upregulated important genes and pathways may play a promoting part in IL-37-regulated immunity and disease.

6.2.3 Conclusion and future directions

Together, growing evidence strongly suggests that IL-37 can not only inhibit but also promote immune cell function. IL-37 does so by upregulating immune activating gene expression and promoting immune cell development and activation. Thus, we believe that IL-37 should be characterised as an immune regulator rather than immune inhibitor.

Whereas the positive impacts of IL-37 on immune cell function is still less understood, it may influence immune response in at least three aspects: promoting certain type of immune response, for instance B cell and M2. Selectively enhancing the function of immune regulatory cell sets, for example, polarising T-reg to enlarge IL-37-mediated immune regulatory effect in immune tolerance; and finally, balancing IL-37-mediated inhibitory effect in the same cell and the same immune context in order to avoid immune deficiency by inducing both immune suppressive and immune active gene expressions. All the hypotheses need to be validated experimentally.

6.3 Role of IL-37 in immunity and disease, friend or foe?

Accumulating reports suggest that IL-37 plays a critical role in a wide range of diseases, including inflammatory, autoimmune, and infectious and tumour disorders (Wang et al, 2018). It has been suggested that IL-37 may be a potential therapeutic drug for the treatment of inflammatory diseases. However, some of the diseases may be exacerbated by IL-37, for instance some infectious diseases. Thus, it is important to be fully aware of the dual effects of IL-37 on disease for better drug development and clinical application. Here I reviewed the IL-37-related publications in PubMed and classified the IL-37-associated diseases into clinical category -allergic, autoimmune, tumour and infectious diseases. I will give a general view of each type of disease categories first, and then focus on the representative and best studied IL-37-regulated diseases in this category as an example to assess the impact of IL-37 on this type of disease. I hope the review and analysis will help to understand the potential beneficial and

detrimental effect of IL-37 on the development and pathogenesis of a variety of diseases.

6.3.1 Allergic disorders

Allergy is an allergen-induced and Th2 cell-mediated hyperactive disorder in which IgE, mast cells and eosinophils play a pathogenic role (Galli and Tsai, 2012). The allergen can be microorganisms, food and chemicals. Allergen can cause a variety of allergic disorders in different organs, for instance, asthma in lung, allergic rhinitis in nose, food allergy in gut and dermatitis in skin (Holgate and Polosa, 2006). There are 40 research and review articles in PubMed by so far that are related to the function of IL-37 in allergic disorders, including allergic asthma, rhinitis and dermatitis (Luo et al, 2014; Charrad et al, 2016; Kim et al, 2017; Lv et al, 2018; Zhu et al, 2018). Importantly, all the reports suggest that IL-37 plays a protective role against the development of allergy by suppressing the development of allergen-induced Th2 response. Allergic asthma perhaps is the most important allergic disorder and the best studied IL-37-regulated allergic condition, therefore I should like to use asthma as an example to see how IL-37 may control allergic response and disease. Asthma is one of the most common chronic inflammatory disorders and a major public health concern. It affects 300 million people worldwide and has unresolved aetiology. It affects more than 5 million people in UK and this number is rising (Holgate et al, 2006; Charrad et al, 2016). It is a complex syndrome characterised by airflow obstruction, bronchial hyper-responsiveness and airway inflammation. Allergic asthma results from multiple pathogenic events involving both genetic and environmental factors (Holgate et al, 2006; Charrad et al, 2016). Dysregulation of function of Th type 2 (Th2) cells that secrete IL-4, IL-5 and IL-13 is thought to play a pivotal role by driving IgE production, recruiting pathogenic cell eosinophils and mast cells to the airway and enhancing their pathogenic functions (Holgate et al, 2006; Charrad et al, 2016). While the detailed mechanisms are still missing, several animal and clinical studies demonstrated that IL-37 is able to inhibit the key pathogenic response in asthma by different mechanisms. It has been shown that IL-37 expression level in asthma patients is low compared to that in healthy controls (McNamee et al, 2011; Zhao et al, 2014). Furthermore, rIL-37 is able to inhibit inflammatory cytokine production in

PBMC and CD4⁺ T cells from asthma patients (Luo et al, 2014; Charrad et al, 2016; Kim et al, 2017; Lv et al, 2018; Zhu et al, 2018).

In an OVA-induced asthma in normal mice, intranasal administration of rIL-37b markedly impaired airway inflammation, mucus production and airway hyperactivity (McNamee et al, 2011). In contrast, the IL-37b failed to protectin asthma mice lacking the IL-37 receptor complex, either IL18R α or IL-1R8, suggesting that the IL-37-mediated effect is receptor dependent (McNamee et al, 2011). Using a house dust mite (HDM)-induced humanized asthma model in NOD/SCID mice, it has been shown that intra venous administration of IL-37b can also suppress the asthma pathogenesis in the airways, including eosinophilia, mucus production, leucocyte infiltration, thickened airway wall (Song et al, 2012). This protective effect mediated by IL-37 in asthma in all the studies is attributed to its immune suppressive effect on Th2 cell function and related cytokine production (IL-4, IL-6, and IL-13) and mast cell as well as the eosinophil functions in these asthmatic contexts (McNamee et al, 2011; Song et al, 2012; Zhao et al, 2014; Godsell et al, 2016). Thus, IL-37b has therapeutic potential for the treatment of asthma. However, the effect of IL-37 on other allergic conditions, for instance food allergy, has not been explored. Furthermore, the function and relative contribution of other IL-37 isoforms to allergic diseases is still unknown. All of these should be studied in the future (Galli et al, 2012).

6.3.2 Autoimmune diseases

Autoimmune diseases are a group of diseases in which dysregulated immune cells target self-cells and organs and cause tissue damage and dysfunction (Rose and Bona, 1993; Walsh and Rau, 2000). Autoimmune diseases can be organ specific and systemic based on one or multiple organs being affected. The common autoimmune diseases are rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), type I diabetes, inflammatory bowel disease (IBD), and psoriasis (Rose and Bona, 1993; Walsh and Rau, 2000). While most autoimmune diseases are with unknown aetiology, abnormality of adaptive immune cell function plays a common pathogenic role in the pathogenesis of the diseases (Rose and Bona, 1993; Singh et al, 2012). T and B-lymphocytes, and particularly T-helper1 (Th1) and Th17 cell-dependent pathways are pivotal in the initiation and persistence of most, if not all, autoimmune diseases (Singh et al, 2012). Th1

cells are polarized by IL-12 and IL-18, which are characterized by selective expression of transcription factor T-bet and IFN γ . Th17 cells are characterised by dominant expression of IL-17 and IL-22 and the transcription factor ROR γ t. The active self-antigen specific T and B cells will cause tissue damage by subsequent activation of tissue specific innate immune cells including macrophage, monocytes and mast cells and the production of proinflammatory mediators (Singh et al, 2012).

Current evidence suggests that IL-37 also plays an anti-inflammatory role in a variety of autoimmune disorders, including rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), systemic lupus erythematosus (SLE), Graves' disease (GD), ankylosing spondylitis (AS), multiple sclerosis (MS) and psoriasis (Singh et al, 2012). In all the reported autoimmune diseases, the serum levels of IL-37 have been measured and compared with the healthy control. In most of the diseases, the IL-37 levels are elevated compared to the healthy controls (Singh et al, 2012; Chen et al, 2014). The levels of IL-37 in the patients, in general, were positively correlated with the parameters of disease activity (Singh et al, 2012; Chen et al, 2014). To understand whether the enhanced IL-37 plays a pathogenic or protective effect in autoimmune diseases, some of the studies further investigated the effect of IL-37 on the inflamed cells ex vivo. The results show that IL-37 can suppress the production of inflammatory cytokines, TNF- α , IL1, IL-6 and IL-17 in the PBMCs from AS, GD, and SLE patients (McInnes and Schett, 2011; Li et al, 2014; El-Barbary et al, 2018). Since IL-37 is inflammatory signals induced, the enhanced IL-37 may represent a feedback inhibition of ongoing inflammation in the patients. However, it may suggest that this enhanced level of IL-37 may be still not enough to control the vigorous inflammatory response in the autoimmune conditions. On the other hand, it is still possible that the enhanced IL-37 may contribute to disease development, as it is positively correlated with disease parameters. More research is needed to answer the question. Nevertheless, I sought next, as an example, to discuss the role of IL-37 in RA in more detail.

Rheumatoid arthritis (RA) is a systemic autoimmune condition. It is the most common inflammatory joint disorder, affecting about 3% world population. Genetic factors, operating together with environmental triggers including

smoking, obesity and unhealthy lifestyle, appear to drive the evolution of disease. It is typically characterised by inflammation and hyperplasia of the synovium, autoantibody production and the destruction of bone and cartilage tissue, which produces considerable pain and disability in severe cases (Singh et al, 2012; Nold- Petry et al, 2015). Moreover, RA sufferers are susceptible to a range of systemic disorders that affects quality of life, including coronary artery disease, stroke and metabolic syndrome arising from the development of insulin resistance.

The T and B-lymphocytes, and particularly Th17 cell-dependent pathways play a pivotal role in the initiation and persistence of RA (Singh et al, 2012; Nold- Petry et al, 2015). Whereas their mode of initiation in the context of RA is currently unknown, Th17 cells may subsequently trigger joint inflammation by activating a wide range of downstream innate effector cells including monocytes and fibroblasts in the synovium and the release of inflammatory cytokines (Singh et al, 2012; Nold- Petry et al, 2015). Several reports show that the levels of IL-37 in serum and cultured PBMCs from RA patients were markedly elevated compared to healthy controls (Singh et al, 2012; Ye et al, 2015). Furthermore, the levels of IL-37 in RA patients were closely associated with the levels of pro-inflammatory cytokines TNF- α , IL-6, IL-17a and the disease severity. This is consistent with the IL-37 levels in AS, GD, and SLE patients. This is also the case in Juvenile Idiopathic Arthritis (El-Barbary et al, 2018). The potential function of IL-37 in the pathogenic Th17 development and function in arthritis has also been studied (Singh et al, 2012).

It has shown that rhIL-37 can effectively reduce the production of IL-17 and Th17 cell numbers in the PBMCs and CD4⁺ T cells in patients with RA (Singh et al, 2012). The reduced Th17 and cytokines number was mainly due to IL-37 inhibiting Th17 cell proliferation, but not Th17 cell differentiation (Singh et al, 2012). In animal RA model, administration of IL-37 delivered by adenovirus system markedly impaired joint inflammation and bone damage. Thus, production of IL-37 in RA may be beneficial. In summary, IL-37 plays a repressing role in several autoimmune diseases tested, suggesting that IL-37 may do so by targeting the key inflammatory pathways including Th17 function and inhibiting the NF- κ B pathways in these diseases (Singh et al, 2012; Nold-Petry et al, 2015).

However, many autoimmune diseases have not been studied and it will be important to know whether if IL-37 has general suppressive effect on all autoimmune diseases. If this is the case, IL-37 may be more valuable to control a wide range of autoimmune disorders.

6.3.3 Malignancies

Cancer still is one of the most serious diseases and a therapeutic challenge in human health (Balkwill and Mantovani, 2001). While the mechanism underlying tumour development is still unclear, inflammation plays a critical role in the initiation, perpetuation and resolution of cancer (Balkwill and Mantovani, 2001; Hagemann et al, 2007). However, current evidence suggests that inflammation is a double-edged sword in tumour development, progression and metastasis (Balkwill and Mantovani, 2001; Karin, 2017). The acute inflammation is thought to be immune protective to remove the mutant and tumour cells by enhancing the function of APC, cytotoxic T cells (CTL) and tumour-specific Th1 cells (Wang and Goldenring; 2002; He and Karin, 2010). However, it has been known that many cancers are developed from chronic inflammation or persistent infections (Balkwill and Mantovani, 2001; Hagemann et al, 2007; Karin, 2017). Several key inflammatory pathways that promote malignancy have been identified, including TLR, nuclear factor κ B (NF- κ B), RONS, Akt and ARRB 1 pathways that promote cell proliferation, survival and mutagenesis (Meira et al, 2008; Tarang et al, 2012; Taniguchi and Karin; 2014). Cytokines also play an important role in the tumour development and progression (Sangaletti et al, 2010; Taniguchi and Karin, 2014). Cytokines such as IL-1, IL-6, and TNF- α are highly expressed in many cancer cells; and promote tumorigenesis and metastasis by stimulating the inflammatory NF- κ B and STAT3 pathways (Sangaletti et al, 2010; Taniguchi and Karin, 2014). Immunosuppressive cells, in particular the FOXP3⁺ regulatory T cells (Treg), also contribute to tumour immune evasion. These cells can abolish anti-tumour immune cell functions by several mechanisms including immunosuppressive cytokine secretion, direct cytotoxicity, and activation of inhibitory receptors (Sakaguchi, 2005; Zou, 2006).

Given its ability in the induction of Treg and suppression of immune cell functions, it has been speculated originally that IL-37 may promote cancer

development by suppressing anti-tumour immune response. However, the current research findings show surprisingly that IL-37 primarily mediates an anti-tumour response in several cancers, including fibrosarcomas, colorectal (CRC), cervical, breast, non-small cell lung cancer (NSCLC) and hepatocellular carcinoma (HCC) (Ding et al, 2016; Abulkhir et al, 2017). IL-37 is expressed in all the tested cancer cells and plays an anti-tumour role in all the studies (Ding et al, 2016; Abulkhir et al, 2017). Here, I should like to focus on three of the best and representative studies to reveal the current understanding of the effect of IL-37b on tumorigenesis in vitro and in animal in vivo.

Fibrosarcomas: The potential therapeutic effect of IL-37b on established fibrosarcoma (A549 lung carcinoma cells) has been studied in animal model in vivo (Gao et al, 2003). A single intratumoral injection of IL-37b-expressing adenovirus into the established fibrosarcoma in mice led to a delayed development and regression of the tumour, the therapeutic effect was more pronounced after multiple injections of IL-37 construct (Gao et al, 2003). While the detailed underlying protective mechanism of IL-37b in cancer is still unknown, it has been shown in the study that IL-37b failed to treat the fibrosarcoma in nude and Severe combined immunodeficiency (SCID) mice without T cells and in IL-12, IFN γ , or Fas ligand-deficient mice (Gao et al, 2003). This is an interesting finding and suggests that IL-37b may kill the cancer cells by inducing a Th1 cell-mediated, Fas-dependent mechanism (Gao et al, 2003). However, the finding is against current belief of IL-37b function as an immune inhibitor, therefore, more solid evidence is needed to support the hypothesis.

Hepatocellular carcinoma (HCC): HCC is a common cancer and mainly developed in patients with chronic hepatitis after hepatitis virus infection or inflammation. The role of IL-37 in HCC has also been investigated (Zhao et al, 2014).

Using immunohistochemical analysis of HCC clinical samples, the authors found that the level of IL-37 in tumour tissues was generally low compared to the normal tissues, and negatively correlated with tumour volumes. Importantly, the HCC patients with higher levels of IL-37 in HCC tumour tissues have better survival rate (Zhao et al, 2014). To understand the potential beneficial effect of IL-37 on HCC, the authors also found that IL-37 levels were correlated with NK but not the CD3 $^{+}$ T cells in the tumour and the overexpression of IL-37 in the

HCC cells enhanced NK cell chemotaxis. In vivo, the HCC cells were transfected with or without IL-37 in lent viral vectors and transplanted into syngeneic mice and then the tumour growth was compared. Consistently, the IL-37 enhanced tumour NK cells infiltration and reduced tumour growth in the mice (Zhao et al, 2014). However, the role of NK cells and the systemic immune response in IL-37-mediated protective effect has not been defined in vivo.

6.3.3.1 Cervical Cancer:

Cervical cancer (CC) is the second most common cancer in women and associated with human papillomavirus (HPV) infection (Sushma et al, 2014). Using mouse cell lines infected (HPV+) or not infected (HPV) with HPV, the role of IL-37 in the proliferation and invasion of CC has been investigated in vitro (Wang et al, 2015). The authors found that overexpression of IL-37 in the cell lines resulted in a significantly reduced tumour cell proliferation and invasion (Transwell invasion assay). Since STAT3 can promote inflammation and cancer, cell proliferation and IL-37b can suppress STAT3 pathway in macrophages (Yu et al, 2009; Nold-Petry et al, 2015). The authors determined the expression and function of STAT3 in IL-37-mediated anti-tumour effect. They found that IL-37 overexpression suppressed STAT3 production and phosphorylation and the production of inflammatory cytokines TNF- α and IL-1 β . Furthermore, knockdown STAT3 using siRNAs abolished IL-37's ability in the reduction of cell proliferation and invasion as well as the expression of TNF- α and IL-1 β (Yu et al, 2009). This result suggests that IL-37 may affect tumour cells expansion and invasion by targeting the key pro-inflammatory pathway STAT3. However, the importance of the IL-37/STAT3-induced anti-tumour effect has not been demonstrated in vivo.

These reports suggest that IL-37 can also suppress tumour growth by different mechanisms and may have impact on treatment and prognostic in human cancers. However, there are some limitations in these studies; all these studies are focused on the direct effect of IL-37 on solid tumours in vitro and vivo. While this is potentially important for therapy, the systemic effect of IL-37 on tumour metastasis and tumour immunity in vivo has not been explored. Since inflammation is a double-edged sword in cancer, it is clinically important to understand and discriminate the IL-37's anti-inflammatory effect on cancer

development and cancer therapy. Furthermore, the effect and importance of other IL-37 isoforms in cancer have not been studied.

6.3.4 Infectious diseases

Notwithstanding the successes of antibiotics and vaccination, pathogenic infection is still a big health problem. This is mainly due to the increasing antibiotic resistant bacteria and the emerging mutant or new strains of pathogens. Some pathogens can also evolutionarily develop the ability to help them to survive and colonise in the human environment by silencing host protective immune mechanisms, for instance the vIL-10 produced by EBV (Kanai et al, 2007).

Immune system plays an essential part in protection against infection. As described in (Chapter 1), during the early infection, innate immune cells, including macrophages, DC and neutrophils can be rapidly activated to control pathogen spread and colonisation in the host. Later, the adaptive immune cells, T and B cells will also be specifically activated, and can eliminate the pathogens by cytotoxicity (CD8 T cells), helping other cells (CD4+ T cells) and producing antibody (B cells). While a rapid and strong pro-inflammatory response helps in pathogen clearance, host tissue destruction secondary to immunopathology can be a deleterious side effect. An appropriate anti-pathogenic response is therefore necessary for minimising tissue damage while still allowing clearance of the invading pathogen. The study of IL-37 in infection has just started and available reports suggest that IL-37 plays a regulatory role in bacterial, virus, parasitic and fungus infections. However, its role in infection is variable depending on the pathogen.

6.3.5 Viral infection

It has been shown that IL-37 can influence the infection and pathogenesis of HBV, HIV and influenza infection (O'Garra, 2013; Højen and Rasmussen, 2015; Zhou et al, 2018). A clinical study involving in 60 HIV-1-infected patients and 20 healthy controls showed that IL-37 expression is associated with levels of inflammatory marker and HIV (Højen and Rasmussen, 2015). IL-37 mRNA levels in PBMCs from patients with HIV were significantly higher than the healthy

controls. The levels of IL-37 mRNA were positively associated with the level of monocyte, but not T, cell markers. In addition, IL-37 expression levels also were correlated with the HIV DNA levels. Thus, the result suggests that IL-37 produced by monocytes may promote HIV replication or infection (Højen and Rasmussen, 2015). However, the real effect on HIV replication has not been revealed.

The expression and function of IL-37 in influenza A virus (IAV) infection was also studied (Zhou et al, 2018). The levels of IL-37 mRNA and protein levels in the PBMC and serum samples from patients with influenza A virus (IAV) infection were enhanced compared to the healthy subjects. To understand the effect of IL-37 on IAV replication in epithelia cells, A549 cell line was infected with IAV first and then treated with rIL-37. The authors found that the titer of IAV RNA was significantly reduced in IL-37 treated cells. The result suggested that IL-37 may be able to inhibit the replication of IAV RNA in infected cells. However, the mechanism underlying the IL-37-reduced virus replication has not been explored.

6.3.6 Bacterial infection

The role of IL-37 in mycobacterium tuberculosis (Mtb), *Strongyloides stercoralis* and pneumococcal pneumonia infection has been reported (Paterson and Orihuela, 2010; Liu et al, 2017; Schauer et al, 2017). Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* and affects one quarter of the world's population. Th1 cells that produce IFN γ play a protective effect on TB (O'Garra, 2013).

A recent report shows that IL-37 may be protective against mycobacterial infection in vitro and in mouse model in vivo (Liu et al, 2017). They found that IL-37 single nucleotide polymorphism (SNP) is closely associated with TB susceptibility. The levels of IL-37 protein in TB patients were elevated compared to levels in healthy controls. Furthermore, Mtb infection enhanced IL-37 but reduced proinflammatory cytokines including IL-6 and TNF- α production in macrophages. In vivo, BCG-infected IL-37-tg mice have less mycobacterial burden and tissue damage in the lung, compared to the infected wt control mice. This suggests that IL-37 can protect against Mtb infection in mice. Moreover, the immune mechanism study demonstrated that the protective effect of IL-37 was associated with an enhanced level of Th1 cells and reduced

regulatory T cells in the spleen (Liu et al, 2017). Thus, IL-37 may protect by promoting anti-bacterial Th1 response and down regulating disease-promoting T-reg response in *Mtb* infection. Unfortunately, how IL-37 promotes Th1 and inhibits Treg response in *Mtb* infection is still unclear and this is controversy in current ideas of IL-37 function.

Streptococcus pneumonia (SP) infections can cause several human diseases, including pneumonia, bacteraemia, and meningitis by enhancing inflammatory pathological response (Paterson and Orihuela, 2010).

Given the effect of IL-37 as a suppressor of innate and acquired immunity, its impact on SP infection has been studied (Schauer et al, 2017). In vitro, the overexpression of IL-37 in the macrophages infected with pneumococci decreased the levels of proinflammatory cytokines IL-6, TNF- α , and IL-1 β , but increased the intracellular pneumococcal infection. Consistently, in vivo, compared to the wt control mice, infection with *S.pneumonia* in IL-37b transgenic mice also decreased the expression of inflammatory cytokines IL-6, TNF- α , and IL-1 β , but enhanced the pneumococcal burden, lung tissue damage and mortality. Thus, IL-37 is a therapeutic target in SP-induced pneumonia.

6.3.7 Helminth infection

The role of IL-37 in parasite infection is still largely unknown. Only two reports from same research group so far show that IL-37 can modulate T cell immune response and cytokine balance in *Strongyloides stercoralis* infection (Anuradha et al, 2015; Breloer and Abraham, 2018).

Strongyloidiasis is a common parasitic disease that affects about 100 million people worldwide (Anuradha et al, 2015; Anuradha et al, 2017; Breloer and Abraham, 2018). The protective immune mechanism in strongyloidiasis in human is largely unknown. The Th1 or Th17 response can be protective or pathogenic depending on the degree of T cell responses. Th2 cell-mediated immune response is thought to play a protective role in the strongyloidiasis by counter-regulation of Th1/Th17-mediated pro-inflammatory response (Anuradha et al, 2015; Anuradha et al, 2017; Breloer and Abraham, 2018). It has been shown that the patients with strongyloides infection contain significantly higher levels of

serum proinflammatory cytokines IFN γ , TNF- α , and IL-1 β and significantly lower levels of anti-inflammatory cytokines IL-4, IL-10, IL-13, IL-27, IL-37, and TGF- β , compared to healthy controls (Anuradha et al, 2015). However, after treatment; the cytokine profiles were reversed with negative enhanced levels of inflammatory cytokines and IL-1 β but reduced anti-inflammatory cytokines including IL-37 (Anuradha et al, 2015). To assess the role of IL-37 in the regulation of CD4 $^{+}$ and CD8 $^{+}$ T cell responses in strongyloidiasis, the authors measured the changes of parasite specific Th1/Tc1, Th2/Tc2, Th9/Tc9, Th17/Tc17, and Th22/Tc22 cell response in the PBMC from the patients with strongyloidiasis and healthy controls following the anti-IL-37 neutralizing antibody treatment in vitro. They found that IL-37 neutralization markedly increased the level of parasite-specific Th1/Tc1, Th2/Tc2, Th17/Tc17, Th9, and Th22 cells by FACS and related cytokines IFN γ , IL-5, IL-9, IL-17, and IL-22 by ELISA in the cultures. Thus, while the reports demonstrate that IL-37 can modulate *S. Stercoralis*-specific T cell response, the precise role of IL-37 in the protective immunity against strongyloidiasis is still unclear (Anuradha et al, 2017).

6.3.8 Fungal infection

There are only two reports in PubMed so far that studied the potential role of IL-37 in candida and paracoccidioidomycosis (Veerdonk et al, 2015). Only the candida has been studied in detail.

Candidiasis is a disease of fungal infection. While the protective immunity against candida infection is still not fully understood, the innate immune cells, in particular the neutrophils in the mucosal tissue, play an important role in anti-candida infection (Anuradha et al, 2017). The influence of IL-37 in candidiasis is unknown. Given its effect on the suppression of neutrophil migration and function, the authors proposed that IL-37 may promote infection by affecting neutrophils function (Veerdonk et al, 2015). They demonstrated the hypothesis in a murine model of disseminated candidiasis using IL-37b transgenic mice. Compared to the wt control mice, IL-37-tg mice were susceptible to candida infection, enhanced fungal growth in the organ and mortality. The mice also failed to effectively produce inflammatory cytokines and recruit neutrophils to the site of infection. Thus, IL-37 may be pathogenic in candidiasis by

interfering with host innate protective inflammatory immune response. However, its effect in human candidiasis has not been explored.

6.4 Conclusion and remark

In summary, IL-37b exhibits a profound effect on the development and perpetuation of a wide range of diseases, including allergic, autoimmune, and infectious and tumour diseases. However, the effect of IL-37 on diseases is varying, and can be beneficial or detrimental.

In allergic diseases tested, IL-37b is generally protective and may be potentially therapeutic in the treatment of allergy and asthma. This is mainly due to its ability to suppress Th2 response and the function of key pathogenic cell sets in allergy, including eosinophils, mast and Th2 cells. It will be important to reveal the effect of IL-37 on other allergic diseases, for instance food allergy, in the future.

IL-37 signal may also be beneficial in the regulation and treatment of several autoimmune diseases by targeting the key inflammatory pathways, including Th1, Th17 and NF- κ B pathways in these diseases. However, its role in the regulation of other autoimmune diseases has not been studied. It will be interesting to know if IL-37 is capable to controlling a wide range of autoimmune disorders.

It is intriguing that IL-37 can also directly suppress the growth of tumour, mainly the solid tumours. Thus, IL-37 may have impact on the treatment and prognostic of human cancers. Future studies should focus on the systemic effect of IL-37 on metastatic tumours and tumour immunity in vivo. The implication of IL-37 on infectious disorders is less clear, particularly the parasite and fungus infection, and likely to be complicated, depending on the pathogen. While this basic and clinical research projects have greatly enhanced our knowledge of the potential application of IL-37 in clinical diseases, the current results are largely generated from animal models. Furthermore, the function and relation of other IL-37 isoforms to diseases is still unknown. All this a waits future should be studied in the future

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